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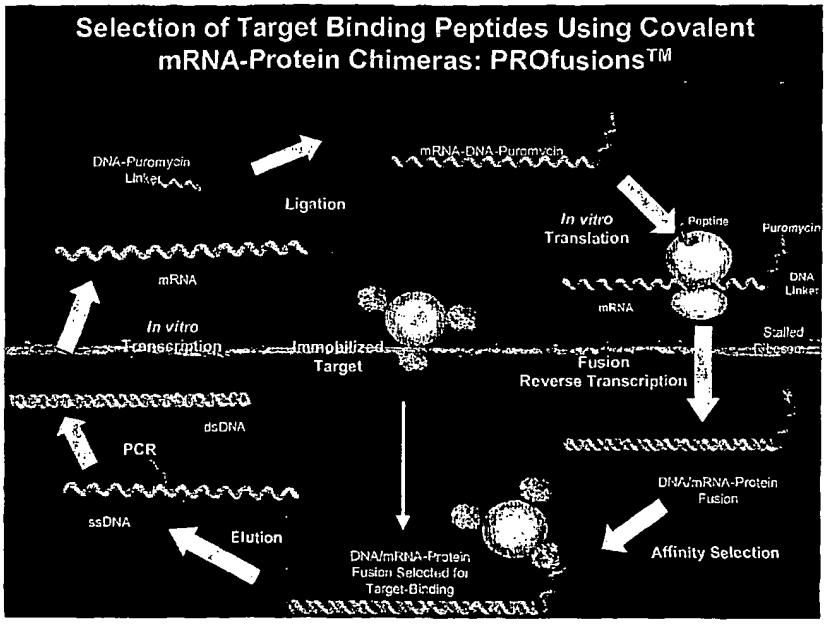

(57) Abstract: The present invention relates to reagents and methods for purifying pertussis toxin (PT).

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METHODS FOR PURIFYING PERTUSSIS TOXIN AND PEPTIDES USEFUL THEREFOR

FIELD OF THE INVENTION

The present invention relates to reagents and methods for purifying pertussis toxin (PT).

BACKGROUND OF THE INVENTION

Pertussis toxin (PT) is produced by *Bordetella pertussis* is a main component in all vaccines against whooping cough. PT is typically combined with tetanus and diphtheria toxoids. Industrial production of PT is typically achieved by cultivating *B. pertussis* in defined media. PT is then isolated from the supernatant and purified by using the well-known techniques (i.e., U.S. Pat. Nos. 6,399,076; 5,877,298; and, Sekura, et al. J. Biol. Chem. 258:14647-14651, 1983; Bogdan, et al. Appl. Env. Micro. 69(10): 6272-6279, Oct. 2003). The majority of known methods each require the use of matrix-bound bovine fetuin (BF) or asialofetuin, the source and purity of which is critical. The use of bovine-derived reagents has led to some concern over bovine-related diseases such as bovine spongioform encephalopathy (BSE).

Those of skill in the art have therefore desired a method for purifying PT that does not rely on BF. One such method is described by Bogdan, et al. (Appl. Env. Micro. 69(10): 6272-6279, Oct. 2003) Peptides having the ability to mimic the glycosidic moiety of bovine fetuin by binding to PT were identified using a phage display system. Three peptides (3G5: NGSFSGF; 3G8: NGSFSGC; and, 3G2: DGSFSGF) having the consensus sequence XGSFSGX (X is any amino acid) were identified as having PT-binding capacity. 3G2 was also utilized in an affinity column to purify PT from a partially purified PT preparation.

Additional methods for designing and utilizing peptides to purify PT in the absence of bovine products are desired by those of skill in the art. Provided herein are reagents and methodologies for affinity purification of PT without the use of fetuin in any form.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. A) Schematic representation of the gurmarin library. Positions of the library that are translated to an amino acid sequence are highlighted. The sequence of the protein portion (59 amino acids in length) is shown in the single letter amino acid code, where X represents

any amino acid. Portions of the library that are not translated are indicated as gray boxes. (a) T7-promoter for optimal *in vitro* transcription of library, (b) TMV – *Tabaco Mosaic Virus* translation initiation sequence for perfect *in vitro* translation of library, (c) His_6-tag for efficient affinity purification of PROfusion™ library, (d) structural, flexible linker, (e) gurmarin core with two randomized loops containing 5 and 9 amino acids respectively, (f) structural, flexible linker and (g) optimized linker for efficient coupling with puromycin-acceptor-molecule. B) The construction of the gurmarin PROfusion™ library is a multi-step process comprising the following reactions: PCR, *in vitro* transcription, chemical ligation of RNA with puromycin-oligonucleotide linker, *in vitro* translation, oligo-dT purification, reverse transcription and His-tag purification.

Figure 2. Schematic representation of a PROfusion™ selection cycle.

Figure 3. Selected gurmarin variants that should be tested for binding activity towards PT. Conserved sequence motifs are highlighted by colored boxes.

Figure 4. Sequence analysis of the gurmarin selection round 4 against PT. The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library and constant regions of the gurmarin scaffold are highlighted. The position of the randomized loops 1 and 2 are indicated.

Figure 5. Sequence analysis of the gurmarin selection round 5a against PT (epoxy). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library and constant regions of the gurmarin scaffold are highlighted. The position of the randomized loops 1 and 2 are indicated.

Figure 6. Sequence analysis of the gurmarin selection round 5b against PT (strep). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library and constant regions of the gurmarin scaffold are highlighted. The position of the randomized loops 1 and 2 are indicated.

Figure 7. Sequence analysis of the gurmarin selection round 6a against PT (strep). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library and constant regions of the gurmarin scaffold are highlighted. The position of the randomized loops 1 and 2 are indicated.

Figure 8. Sequence analysis of the gurmarin selection round 6b against PT (strep). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library and constant regions of the gurmarin scaffold are highlighted. The position of the randomized loops 1 and 2 are indicated.

Figure 9. Selected PP26 variants that will be tested for binding activity towards PT. Conserved sequence motifs are highlighted.

Figure 10. Sequence analysis of the PP26 selection round 4 against PT. The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library are highlighted.

Figure 11. Sequence analysis of the PP26 selection round 5a against PT (epoxy). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library are indicated by light yellow boxes. Conserved sequence motifs are highlighted.

Figure 12. Sequence analysis of the PP26 selection round 5b against PT (strep). The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library are indicated by light yellow boxes. Conserved sequence motifs are highlighted.

Figure 13. Sequence analysis of the PP26 selection round 6a against PT. The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library are indicated by light yellow boxes. Conserved sequence motifs are highlighted.

Figure 14. Sequence analysis of the PP26 selection round 6b against PT. The amino acid sequence of individual variants is shown in the single letter amino acid code. Constant, flanking regions of the library are highlighted.

Figure 15. Immobilization of synthetic biotinylated core peptides to Streptavidin sepharose and verification of binding to purified PT. The unbound fraction of PT was analyzed by separation of 1/40 volume of the supernatant after binding on a 12 % NuPage gel with MES running-buffer (upper gel). To analyze sepharose bound PT 50% of the eluate was separated on 12 % NuPage gel with MES running-buffer (lower gel). Detection was performed by silver staining. Defined amounts of purified PT were used as standard for quantification, except for the gurmarin peptides 15 and 9.

Figure 16. Purification of PT out of Sample A (left gel) and Sample B (right gel). To analyze sepharose bound PT 50% of the eluate was separated on 12% NuPage gel with MES running-buffer (lower gel). Detection was performed by silver staining. Defined amounts of purified PT were used as standard for quantification, except for the gurmarin peptide 9.

Figure 17. Optimization of the washing conditions of bound PT out of sample A or B to immobilized peptides pp26 clone 9 and 15 and gurmarin clone 9 and 15 using 3 washes of 50

mM Tris/HCl, pH 7.5 or 50 mM acetate, pH 6. The PT were analyzed on 12% Bis Tris gels and visualized by silver staining. PPM: protein perfect marker.

Figure 18. Optimization of washing conditions of bound PT out of sample B to immobilized peptides pp26 clone 9 using 3 to 20 washes of 50 mM Tris/HCl, pH 7.5 or 50 mM acetate, pH 6. The PT was analyzed on 12% Bis Tris gels and visualized by silver staining.

Figure 19. Elution of PT from peptide streptavidin sepharose with 0.2 to 2.0 M$MgCl_2$ in 50 mM Tris/HCl. Peptide bound PT was displaced from the peptide-streptavidin sepharose by three consecutive washes with the indicated elution buffers (20 µl each). Remaining material was subsequently eluted with gel loading buffer. All elutions were analyzed on 12% Bis Tris gels (1x MES running buffer) and visualized by silver staining.

Figure 20. Elution of PT from peptide streptavidin sepharose under acidic (50 mM ~~glycin~~ glycine, pH 2.5) or basic (100 mM carbonat boffer, pH 10.5) conditions. Peptide bound PT was displaced from the peptide streptavidin sepharose (20 µl containing ~200 pmol of one peptide) by three consecutive washes with with the indicated elution buffers (40 µl each). Remaining material was subsequently eluted with gel loading buffer. All elutions were analyzed on 12% Bis Tris gels (1x MES running buffer) and visualized by silver staining. 1/40 volume of the flow through after peptide streptavidin sepharose incubation with sample A was analyzed was analyzed on the same gel for each peptide.

Figure 21. Small Scale column purification of PT from sample B on streptavidin sepharose with immobilized pp26 peptide 9 as affinity ligand (A) an gel estimation of the yield of purified PT (B).

Figure 22. Small Scale column purification of PT from sample B on streptavidin sepharose with immobilized gurmarin peptide 15 as affinity ligand (A) an gel estimation of the yield of purified PT (B).

Figure 23. PT binding to peptide streptavidin sepharose in dependence of varying amounts of peptide (as indicated) used for immobilzation on streptavidin sepharose (per 1 ml). Amount of bound PT was quantified by direct comparison to defined amounts of purified PT on the same gel. As an example, pp26/9 is plotted against the amount of peptide used for immobilization per ml of streptavidin sepharose. Maximal binding was estimated at approximately 100-150 pmol PT.

Figure 24. PT yield as function of varying amounts of input material (sample B) per µl peptide streptavidin sepharose or 6.85 µl asialofetuin sepharose. The amount of eluted PT was calculated on the basis of direct comparison to defined amounts of purified PT on the same gel and listed in the **Table 12.**

Figure 25. Reutilization of peptide sepharose for repeated PT binding and elution. Bound PT to streptavidin sepharose were 4 times eluted with 100 mM Carbonate buffer at pH 10.5 and the cloumn matrix was regenerated with 10 mM HCl.

Figure 26. PT elution fractions after FPLC-column purification on pp26/9 peptide streptavidin sepharose (0.5 ml) from sample B. The elution fractions (0.5 µl of each 500 µl elution) were analyzed by PAGE (12% Bis-Tris-Gel, MES running buffer) and silver staining. Defined amounts of purified PT were seperated on the same gel for direct comparision. Concentration of PT was determined by measuring the absorbance of the elution fractions at 280 nm (A_{280}) and compared to purified PT standards (see table).

SUMMARY OF THE INVENTION

The present invention relates to methods for purifying pertussis toxin (PT). In one embodiment, a method for generating a DNA-protein fusion by covalently bonding a nucleic acid reverse-transcription primer bound to a peptide acceptor to an RNA, translating the RNA to produce a peptide product such that the protein product is covalently bound to the primer, reverse transcribing the RNA to produce a DNA-protein fusion, and testing the fusion product to identify those containing PT binding peptides. The sequence of the peptide is then identified by sequencing. In other embodiments, peptides are provided that have PT-binding capacity and are useful for purifying PT from complex biological fluids. Also provided are peptides bound to solid supports and/or chromatographic media for use in purifying PT from complex biological fluids and methods for carrying out such purifications.

DETAILED DESCRIPTION

The present invention provides reagents and methodologies for a new method for purifying pertussis toxin (PT). As described above, one such method has been demonstrated by Bogdan, et al. In that method, phage display was utilized to identify PT-binding peptides. For the purposes of practicing the present invention, PT includes naturally expressed PT, detoxified PT (genetically or otherwise), natural or other PT variants, recombinant PT, PT fragments, or other versions of PT (see, for example, U.S. Pat. Nos. 6,399,076; 6,168,928; 6,018,022; 5,977,304; 5,965,385; 5,856,122; 5,877,298; 5,433,945; 5,358,868; 5,332,583; 5,244,657; 5,221,618; 5,085,862; 4,997,915). In most cases, chemical detoxification is performed following purification of PT. Any form of PT is suitable for use in practicing the present invention as long as a reagent as described herein has the ability to bind the particular

form of PT. Within this application, all cited references, patents, and patent applications are incorporated herein by reference.

The present invention also relates to the use of recombinant technology to identify PT-binding peptides. The present invention provides advantages over methods already known in the art. In addition, novel peptides useful in purifying PT are provided herein. In one embodiment, a method for generating a DNA-protein fusion by covalently bonding a nucleic acid reverse-transcription primer bound to a peptide acceptor to an RNA, translating the RNA to produce a peptide product such that the protein product is covalently bound to the primer, reverse transcribing the RNA to produce a DNA-protein fusion, and testing the fusion product to identify those containing PT binding peptides. The sequence of the peptide is then identified by sequencing. In certain embodiments, the RNA moiety may be removed from the complex by treatment with an RNA-degrading compound such as RNase H. Photocrosslinking reagents and peptide acceptors are also useful in practicing the present invention. This system and related reagents have been described elsewhere in, for example, U.S. Pat. Nos. 6,416,950 (Lohse, et al); 6,429,300 (Kurz, et al.); 6,436,665 (Kuimelis, et al.); 6,602,685 (Lohse, et al); and, 6,623,926 (Lohse, et al).

In practicing the invention, a reagent such as a nulceic acid, peptide, fusion, ligand, affinity complex, or the like may be non-diffusively bound or attached to a solid support. In order to be non-diffusively bound or attached, the reagent is chemically or physically combined with the solid support such that the reagent does not move in the presence of liquid from a region of high concentration of reagent to a region of low concentration of reagent. A solid support is any column (i.e., unpacked or packed chromatographic media, column material), bead, test tube, microtiter dish, solid particle (i.e., agarose or sepharose), microchip (i.e., silicon, silicon-glass, or gold chip), membrane (i.e., the membrane of a liposome or vesicle), or other medium to which a reagent may be bound or attached, either directly or indirectly (for example, through other binding partner intermediates such as an antibody, Protein A, Protein G, streptavidin, biotin).

In preferred embodiments, the reagent is a substance or compound having the ability to bind PT. More preferably, the reagent is a substance or compound having the ability to reversibly bind PT. Even more preferably, the reagent is a peptide having the ability to at least bind, and preferably reversibly bind PT within a liquid containing components other than PT. A reagent that reversibly binds PT is one that binds PT under certain conditions (adsorption), and releases PT under other conditions (desorption). For example, the reagent may bind PT when exposed to conditions of neutral pH and release PT following exposure to

conditions of acidic or basic pH. Thus, the ability of the reagent to bind PT (i.e., the equilibrium dissociation constant or K_d) may be manipulated by altering the conditions under which the reagent is in contact with PT. Other conditions may also be changed, including temperature, ionic strength (i.e., concentration of an ionic salt such as sodium chloride or magnesium chloride, for example), solvent concentration, presence or absence of a competitor reagent / free ligand / analogue, polar properties, among others as is known in the art.

In certain embodiments, an affinity matrix (i.e., a PT-binding peptide bound to a solid support) is utilized to separate a desired component (i.e., PT) from a complex mixture found within a liquid, biological or otherwise. In certain cases, it may be desirable to purify PT from a complex biological fluid such as a bacterial lysate or other composition in which PT does not comprise the majority of components within the fluid (as determined by SDS-PAGE, for example). In other cases, PT may be isolated from a composition that has been partially purified for PT such that the majority of the components within the fluid is represented by PT (a composition consisting of approximately greater than or equal to 50% PT). For example, a composition in which PT consists of about 50% or more of the total protein in the composition as determined by SDS-PAGE would under most circumstances be considered partially purified.

To purify PT, a composition containing PT may be placed into contact with a PT-binding reagent, preferably a reversibly binding PT-binding reagent, that is bound to a solid support for a sufficient period of time such that PT and the PT-binding reagent bind to one another to form a complex. Non-PT components are then washed away. One or more conditions (i.e., pH) are then changed such that the K_d of the PT-PT binding reagent bond increases, and PT is released from the complex. Released PT is then collected and prepared for further use. Such a separation may be termed affinity purification and products so purified referred to as being affinity purified.

Chromatographic techniques that are generally considered by those of skill in the art to be less selective than affinity purification techniques may also be used in practicing the present invention. As is known in the art, such techniques may include, for example, size-exclusion chromatography, ion-exchange chromatography, reverse-phase chromatography, and hydrophobic-interaction chromatography. Any of these techniques (including affinity purification) may be carried out using the proper solid support in a low pressure chromotography (LPC), high pressure liquid chromotography (HPLC), or fast protein liquid chromotography (FPLC) setting, for example. Suitable solid supports and equipment for carrying out such techniques are widely available in the art. In practicing the present

invention, both affinity chromatography and the more generalized techniques may be combined as needed to either partially purify a starting material (i.e., complex biological fluid such as a bacterial lysate), purify material, or further purify affinity- or otherwise-purified material (i.e., affinity purified PT).

Peptides have been identified that bind PT and are described herein. Certain peptides have been found to bind PT with high affinity. Such preferred PT binding peptides include:

RSSHCRHRNCHTITRGNMRIETPNNIRKDA (pp26-5);

STMNTNRMDIQRLMTNHVKRDSSPGSIDA (pp26-6);

RSNVIPLNEVWYDTGWDRPHRSRLSIDDDA (pp26-9);

RSWRDTRKLHMRHYFPLAIDSYWDHTLRDA (pp26-15);

SGCVKKDELCARWDLVCCEPLECIYTSELYATCG (G-9);

SGCVKKDELCELAVDECCEPLECFQMGHGFKRCG (G-10);

SGCVKKDELCSQSVPMCCEPLECKWFNENYGICGS (G-15); and,

SGCVKKDELCELAIDECCEPLECTKGDLGFRKCG (G-19).

Of these, especially preferred peptides include:

RSNVIPLNEVWYDTGWDRPHRSRLSIDDDA (pp26-9); and,

SGCVKKDELCSQSVPMCCEPLECKWFNENYGICGS (G-15).

Further contemplated are related peptides such as, for example, fragments, variants orthologs, homologues, and derivatives, for example, that possess at least one characteristic or activity (i.e., activity, antigenicity) of the peptide. A fragment comprises a truncation of the sequence (i.e., nucleic acid or polypeptide) at the amino terminus (with or without a leader sequence) and / or the carboxy terminus of the peptide. Fragments may also include variants, orthologs, homologues, and other variants having one or more amino acid additions or substitutions or internal deletions as compared to the parental sequence. In preferred embodiments, truncations and/or deletions comprise about one amino acid, two amino acids, five amino acids, 10 amino acids, 20 amino acids, 30 amino acids, 40 amino acids, 50 amino acids, or more. A variant is a sequence having one or more sequence substitutions, deletions, and/or additions as compared to the parental sequence. Variants may be naturally occurring or artificially constructed. Such variants may be prepared from the corresponding nucleic acid molecules. In preferred embodiments, the variants have from 1 to 3, or from 1 to 5, or from 1 to 10, or from 1 to 15, or from 1 to 20, or from 1 to 25, or from 1 to 30, or from 1 to 40, or from 1 to 50, or more than 50 amino acid substitutions, insertions, additions and/or deletions.

Substitutions may be conservative, or non-conservative, or any combination thereof. Conservative amino acid modifications to the sequence of a polypeptide (and the corresponding modifications to the encoding nucleotides) may produce polypeptides having functional and chemical characteristics similar to those of a parental polypeptide. For example, a "conservative amino acid substitution" may involve a substitution of a native amino acid residue with a non-native residue such that there is little or no effect on the size, polarity, charge, hydrophobicity, or hydrophilicity of the amino acid residue at that position and, in particlar, does not result in decreased immunogenicity. Suitable conservative amino acid substitutions are shown in **Table I.**

Table I

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln	Gln
Asp	Glu	Glu
Cys	Ser, Ala	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

A component such as PT may be said to be purified when it has been separated from at least about 50% of the proteins, lipids, carbohydrates, or other materials with which it is originally found (i.e., a bacterial lysate). It is preferred that the component be separated from at least about 95-100%, 90-95%, 80-90%, 70-80%, 60-70% or 50-60% of the total protein content of a composition as determined by SDS-PAGE, for example. In certain embodiments, a purified component is one that is useful in inducing an immune response in a host to whom the component has been administered, either alone or in combination with other agents. The

immune response may include the production of antibodies that bind to at least one epitope of PT or *Bordetella pertussis*, for example, and / or the generation of a cellular immune response against cells expressing PT. The response may be an enhancement of a current immune response by, for example, causing increased antibody production, production of antibodies with increased affinity for the antigen, or an increased cellular response (i.e., increased T cells). Other measures of an immune reponse are known in the art and would be suitable in determining whether or not an immune response has occurred.

PT isolated using the methods described herein may be prepared as pharmaceutical compositions. Preferred pharmaceutical compositions include, for example, PT in a liquid preparations such as a suspensions, syrups, or elixirs. Preferred injectable preparations include, for example, peptides suitable for parental, subcutaneous, intradermal, intramuscular or intravenous administration such as sterile suspensions or emulsions. For example, PT may be prepared as a composition in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The composition may also be provided in lyophilized form for reconstituting, for instance, in isotonic aqueous, saline buffer. Such compositions may also be prepared and utilized as a vaccine as described in, for example, U.S. Pat. No. 5,877,298 and 6,399,076 (Vose, et al.) as well as International App. No. PCT/CA96/00278. PT prepared as indicated herein may also be combined with other antigens from disease-causing organisms such as Corynebacterium (i.e., diphtheria), Clostridium (i.e., tetanus), polio virus (i.e., IPV, OPV), hepatitis virus, Neisseria (i.e., meningitis), Streptococcus, Hemophilus, or other pertussis antigens (i.e., filamentous ~~hemaglutinin~~ hemagglutinin, pertactin, and agglutinogens), among others as is known in the art.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

Materials and Methods

A. Pertussis Toxin (PT)

PT is a heterooligomeric protein complex with a MWr of 109 kD (consists of the 6 subunits S1, S2, S3, 2x S4, S5). A high purity (> 99.99%) preparation, formulated as an ammonium sulfate precipitate, was utilized. A PT-specific ligand (asialofetuin) recognizing the native hexameric complex was also utilized. Asialofetuin is available in a solubilized and in sepharose immobilized form.

B. Gumarin Library Selection

Gurmarin is a 35-residue polypeptide from the Asclepiad vine *Gymnea sylvestre*. It has been utilized as a pharmacological tool in the study of sweet-taste transduction because of its ability to selectively inhibit the neural response to sweet tastants in rats. It has no apparent effect in humans. It has been suggested that the taste-suppressing of gurmarin might be due to the peptide either by binding directly to a sweet-taste receptor or interacting with a downstream target in the sweet-taste-transduction system ~~(1)~~.

Gurmarin belongs to the family of "knottins", a group of structurally related proteins, typically less than 40 residues in length. Knottins bind to a diverse range of molecular targets that includes proteins, sugars and lipids but share a common scaffold comprising a small triple-stranded antiparallel β-sheet and disulphide bound framework ~~(2, 3)~~.

A specialized gurmarin-library was designed with 15 randomized amino acid positions, as shown below:

```
Wild-type gumarin: qqCVKKDELCIPYYLDCCEPLECKKVNWWDHKCig
Gumarin core:        CVKKDELCXXXXXXCCEPLECXXXXXXXXXC
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Within the gumarin core sequence, X represents any amino acid. This library was validated to yield high affinity binders against protein targets. The gurmarin library combines a set of advantages that makes it the best choice for a selection against the PT-toxin for at least the following reasons: limited flexibility makes up for high entropic cost in conforming to target topology; theoretically fewer amino acids for higher affinities than in linear libraries; resistant to proteases; and susceptibility to redox-elution conditions in downstream applications. The gurmarin library was constructed using process shown in **Figure 1.**

1. PCR of starting oligonucleotides

Three gel-purified oligos were used to construct the gurmarin library with two randomized loops. 1 nmole of gurmarin template (≈ ca. $6 \cdot 10^{14}$ sequences) 5'- AGT GGC TCA AGC TCA GGA TCA GGC TGC GTC AAG AAA GAC GAG CTC TGC NNS NNS NNS NNS NNS NNS TGC TGT GAG CCC CTC GAG TGC NNS NNS NNS NNS NNS NNS NNS NNS NNS TGC GGC AGC GGC AGT TCT GGG TCT AGC-3', was amplified for 6 rounds of PCR (94°C, 1 min; 65°C, 1 min; 72°C, 1 min) using 1 μM of the 5'-His-Tag Primer 5'-TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT ACA ATG CAC CAT CAC CAT CAC CAT AGT GGC TCA AGC TCA GGA TCA-3' and 1 μM of the 3'-Primer 5'-TTT TAA ATA GCG GAT GCT ACT AGG CTA GAC CCA GAA CTG CCG CT-

3' using *Taq*-polymerase and analyzed on a 2 % agarose gel, which indicated a representative library had been constructed.

2. *In vitro transcription*

dsDNA was transcribed into RNA using the RiboMax Express *In vitro* transcription kit from Promega. After incubation for 45 min at 37°C, DNase I was added and the incubation at 37°C continued for an additional 15 minutes. This mixture was subjected to a phenol/chloroform extraction. Excess of NTPs was removed by NAP-5 gel filtration (Pharmacia). RNA was analyzed on a 6%-TBU-gel, and incidacted that the dsDNA had been efficiently transcribed.

3. *Chemical coupling of RNA and puromycin-oligonucleotide linker*

Purified RNA will be annealed (85°C, 1 min ~~-~~ cool down to 25°C at a ramp of 0.3°C/s) to a 1.5-fold excess of puromycin-oligonucleotide linker PEG2A18: 5'-psoralen-*UAG CGG AUG C* A_{18} $(PEG\text{-}9)_2$ CC puromycin (nucleotides shown in italics represent 2'-O-methyl-derivatives). The covalent coupling is performed by illumination for 15 min at RT (RT) with UV-light (365 nm). The reaction product was analyzed on 6%-TBU gel and indicated the linking reaction had proceeded efficiently.

4. *In vitro translation*

Ligated RNA was translated using the rabbit reticulocyte lysate from Promega in the presence of 15 µCi ^{35}S-methionine (1000 Ci/mmole). After a 30 min incubation at 30°C, KCl and $MgCl_2$ were added to a final concentration of 530 mM and 150 mM respectively and a sample was analyzed on 4-20% Tris/glycine-SDS-PAGE. The gel indicated that the translation reaction was successful.

5. *Oligo-dT purification*

Molecules (mRNA-protein fusions) were isolated by incubation with oligo dT magnetic beads (Miltenyi) in incubation buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM NaCl and 0.25 % Triton X-100) for 5 min at 4°C. PROfusion™ molecules were isolated by filtration through MiniMACS-columns (Miltenyi), washing with incubation buffer and elution with water. A sample was analyzed on 4-20% Tris/glycine-SDS-PAGE, and indicated that the reaction was successful.

6. Reverse transcription

A corresponding cDNA strand was generated by reverse transcription with SuperScript II Reverse Transcriptase (Gibco BRL) under the manufacture's recommended conditions using a 5-fold excess of 3'-Primer. A sample was analyzed on 4-20% Tris/glycine-SDS-PAGE, and indicated that the reaction was successsul.

7. His-tag purification

Reverse transcribed PROfusion™ molecules were mixed with Ni-NTA-agarose (50 µl/10 pmole PROfusion™) (QIAGEN) in HBS buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X-100, 100 µg/ml sheared salmon sperm DNA, 1 mg/ml BSA) and incubated for 60 min at RT under gentle shaking. Ni-NTA was then filtrated, washed with HBS/5 mM imidazole and PROfusions™ were eluted with HBS/150 mM imidazole. A sample was analyzed on 4-20% Tris/glycine-SDS-PAGE, and indicated that the purification was successful. 20 pmole (≈ ca. $1 \cdot 10^{13}$ sequences) of PROfusion™ molecules will be used as input for each selection.

B. Linear peptide library PP26 for selection

A specialized linear peptide library PP26 with 26 randomized amino acid positions was also designed using the following construct:

T7-TMV-MGRGS-HHHHHH-ARS-XXXXXXXXXXXXXXXXXXXXXXXXXX-DANAPK-ASAI

The sequence of the protein portion (50 amino acids in length) is shown in the single letter amino acid code, where X represents any amino acid. Portions of the library that are not translated include: (a) T7: the T7-promoter for optimal *in vitro* transcription of library; and, (b) TMV: the *Tabaco Mosaic Virus* translation initiation sequence for perfect *in vitro* translation of library. MGRGS represents a structural, flexible linker. HHHHHH represents a His_6-tag for efficient affinity purification of PROfusion™ library. ARS represents a second structural, flexible linker. DANAPK represents a third structural, flexible linker. ASAI represents an optimized linker for efficient coupling with puromycin-acceptor-molecule.

This library was validated to yield high affinity binders against protein targets. The PP26 library combines two major advantages that makes it an excellent choice for the selection of chromatographic affinity reagents: high flexibility: can conform to the topology of the target; and robustness due to the absence of a conserved structure the resulting binders are resistant to harsh biophysical conditions

1. PCR of starting oligonucleotides

Three gel-purified oligos were used to construct the gurmarin library with two randomized loops. 1 nmole of PP26 template (≈ ca. $6 \cdot 10^{14}$ sequences) 5'-AGC GGA TGC CTT CGG AGC GTT AGC GTC SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN SNN AGA TCT AGC ATG ATG ATG ATG A-3', was amplified for 6 rounds of PCR (94°C, 1 min; 65°C, 1 min; 72°C, 1 min) using 1 µM of the 5'-His-Tag Primer 5'-TAA TAC GAC TCA TAG GGA CAA TTA CTA TTT ACA ATT ACA ATG GGA CGT GGC TCA CAT CAT CAT CAT CAT CAT GCT AGA TCT -3' and 1 µM of the 3'-Primer 5'-AA TTA AAT AGC GGA TGC CTT CGG AGC GTT AGC -3' using *Taq*-polymerase and confirmed by analysis on a 2 % agarose gel.

2. In vitro transcription

dsDNA was transcribed into RNA using the RiboMax Express *In vitro* transcription kit from Promega. After incubation for 45 min at 37°C, DNase I was added and the incubation at 37°C continued for an additional 15 minutes. This mixture was subjected to a phenol/chloroform extraction. Excess of NTPs was removed by NAP-5 gel filtration (Pharmacia). Transcription of RNA was confirmed by analysis on a 6%-TBU-gel.

3. Chemical coupling of RNA and puromycin-oligonucleotide linker

Purified RNA will be annealed (85°C, 1 min ∧ cool down to 25°C at a ramp of 0.3°C/s) to a 1.5-fold excess of puromycin-oligonucleotide linker PEG2A18: 5'-psoralen-*UAG CGG AUG C* A_{18} $(PEG\text{-}9)_2$ CC puromycin (nucleotides shown in italics represent 2'-O-methyl-derivatives). The covalent coupling is performed by illumination for 15 min at RT (RT) with UV-light (365 nm). The reaction was confirmed by analysis of the reaction product on 6%-TBU gel.

4. In vitro translation

Ligated RNA was translated using the rabbit reticulocyte lysate from Promega in the presence of 15 µCi ^{35}S-methionine (1000 Ci/mmole). After a 30 min incubation at 30°C, KCl and $MgCl_2$ were added to a final concentration of 530 mM and 150 mM respectively and translation confirmed by analysis on 4-20% Tris/glycine-SDS-PAGE.

5. Oligo-dT purification

Molecules (mRNA-protein fusions) were isolated by incubation with oligo dT magnetic beads (Miltenyi) in incubation buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM NaCl and 0.25 % Triton X-100) for 5 min at 4°C. PROfusion™ molecules were isolated by filtration through MiniMACS-columns (Miltenyi), washing with incubation buffer and elution with water. A sample was analyzed to confirm the reaction on 4-20% Tris/glycine-SDS-PAGE.

6. Reverse transcription

A corresponding cDNA strand was generated by reverse transcription with SuperScript II Reverse Transcriptase (Gibco BRL) under the manufacture's recommended conditions using a 5-fold excess of 3'-Primer. A sample was analyzed to confirm transcription on 4-20% Tris/glycine-SDS-PAGE.

7. His-tag purification

Reverse transcribed PROfusion™ molecules were mixed with Ni-NTA-agarose (50 µl/10 pmole PROfusion™) (QIAGEN) in HBS buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X-100, 100 µg/ml sheared salmon sperm DNA, 1 mg/ml BSA) and incubated for 60 min at RT under gentle shaking. Ni-NTA was then filtrated, washed with HBS/5 mM imidazole and PROfusions™ were eluted with HBS/150 mM imidazole. A sample was analyzed to confirm the reaction on 4-20% Tris/glycine-SDS-PAGE. 20 pmole (≈ ca. $1 \cdot 10^{13}$ sequences) of PROfusion™ molecules will be used as input for each selection.

C. Target Preparation

In the PROfusion™ technology highly diverse substance libraries, which are composed of up to 10^{13} different PROfusion™ molecules (mRNA-Protein fusions), are selected against a wanted target (protein, sugar or lipid) for high affinity binding. In this process the targets will typically be immobilized to solid phases. These solid phase are preferentially magnetic beads that allow fast and efficient handling during the selection process and give low background.

1. Test targets for nuclease activity

Targets - 5 µg PRP and 0.5 µg PT - were contacted with 0.12 pmole radioactive labeled PROfusion™ library molecules at 4°C and RT (RT) followed by an incubation for 1 h

and 16 h respectively. The integrity of PROfusion™ molecules after incubation was confirmed by 4-20% Tris/glycine SDS-PAGE and subsequent autoradiography. Degradation of PROfusion™ molecules was not detected, thus demonstrating that the targets are free of nucleases.

2. Test targets for protease activity

Targets - 5 µg PRP and 0.5 µg PT - were contacted with 1 µg purified GST-protein at 4°C and RT followed by an incubation for 1 h and 16 h respectively. The integrity of GST-protein after incubation was analyzed by 4-20% Tris/glycine SDS-PAGE and subsequent Coomassie Brilliant Blue staining. Degradation of GST-protein was not detected, thus demonstrating that the targets are free of proteases.

D. Immobilization of PT

1. Reconstitution of PT

500 µl of the precipitate (2,26 mg/ml) as delivered by Aventis Pasteur were centrifuged at 21.400xg for 45 min at RT. The supernatant was discarded; the pellet was dissolved in 1100 µl CTW-buffer (0.286 g $NaHCO_3$, 0.170 g Na_2CO_3, 50 µl Tween-80, add to 50 ml MilliQ H_2O). To check the quality of this PT preparation a dilution series (250 ng, 500 ng, 1 µg, 2.5 µg, 5 µg and 15 µg) was separated on a 4-12 % BisTris SDS-PAGE, run in MES-buffer). At least 4 bands could be clearly separated, corresponding to the subunits S1 (28 kD), S2 (23 kD), S3 (22 kD) and S4 (11.7 kD). The smallest protein S5 (9.3 kD) in the PT-complex could not be seen. Probably, this band co-migrates in this gel system with the only slightly larger S4 subunit.

2. Coupling strategy

Several methods were established for immobilization of proteins to magnetic particles. In principle two major strategies are used: primary amino groups and sulfhydryl groups of the target protein are tethered covalently to epoxy-activated magnetic beads (Dynal) forming stabile amide or thioether bounds. This reaction is performed in the presence of ammonium sulfate to promote the reaction and typically results in a very efficient coupling of the target protein. Anyhow, certain proteins seem to undergo structural changes under these conditions resulting in a bound but not native and/or inactive conformation; and, primary amino groups and sulfhydryl groups of the target protein are tethered covalently to NHS-ester activated biotin derivatives (Pierce) subsequently followed by an immobilization of now biotinylated protein to streptavidin magnetic beads (Dynal)

Typically, covalent coupling of a target protein to epoxy beads is preferred if reaction conditions are suitable for a given target since this method guarantees that only the target is presented on the beads. In the case of a biotin/streptavidin coupling the beads also present streptavidin that could lead to the enrichment of anti-streptavidin binder during a selection. Therefore, Phylos has established specialized methods to preclear PROfusion™ libraries for streptavidin binders to get high quality results for a given target. But in total a covalent coupling typically results in a faster enrichment of target specific binders. In the specific case of PT it is most reasonable to start with a covalent coupling strategy since it is known that ammonium sulfate incubation does not influence the functionality of the PT-protein.

3. Optimization of coupling conditions to epoxy beads (Dynal)

The coupling conditions for PT were optimized in several independent experiments (different ammonium sulfate concentrations (0.5 – 2.0 M) and different beads/target-ratios were applied, as well as time- and temperature dependency (2 min – 16 h; 8 °C - RT). Best results were observed for the following reaction condition: A final volume of 300 µl, consisting of 100 µg PT, $3.3 \cdot 10^8$ beads and a final ammonium sulfate concentration of 1M was incubated in a time course for 2 min to 60 min at RT in a 2 ml Eppendorf tube. After incubation the tube was placed in a magnet for 4 min to pull down the beads and the supernatant was stored for subsequent gel analysis. The beads were washed once with 1 ml HEPES-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X100) and an aliquot of beads (5 % of the beads) were analyzed on a 4-12% BisTris SDS-PAGE to determine the amount of associated protein. It was found that coupling of PT to epoxy beads occurs very efficiently even after only a two minute reaction.

4. Semi-preparative coupling of PT to epoxy beads

2.6 mg dry epoxy-activated beads (M-270, Dynal) (~ $1.7 \cdot 10^8$ beads) were resuspended in 1 ml phosphate buffer (19 mM NaH_2PO_4, 81 mM Na_2HPO_4, pH 7.4) and equilibrated for 10 min. The equilibration was repeated two times with fresh phosphate buffer. Subsequently the beads were directly used in a coupling reaction with 480 pmole reconstituted PT (1 µg/µl in CTW buffer) in 1 M ammonium sulfate (final volume 157 µl). After incubation at RT for 15 min under continuos agitation the beads were washed with 300 µl HBS-buffer, followed by three washing steps with HEPES-buffer and finally resuspended in 240 µl HEPES-buffer and stored in aliquots at 4°C. The effectiveness of the coupling reaction was checked by a SDS-

polyacrylamidgel-analysis of all wash fractions, the supernatant of the coupling reaction and the fraction of PT which was removable from the washed beads by SDS-loading-buffer.

5. *Analysis of epoxy-bead immobilized PT for its binding to asialofetuin*

40 µl of the PT-derivatized beads were incubated with 320 pmole asialofetuin in HEPES-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton-X100) for 1 h at RT (final reaction volume 200 µl), washed 2-7 times with 200 µl HEPES-buffer and finally resuspended in 30 µl HEPES-buffer. 50% of the beads were analyzed on SDS-PAGE to confirm the reaction.

Tests of these PT-derivatized beads after one week of storage at 4°C showed a reduced asialofetuin binding capacity indicating that the material looses its performance by long term storage. Thus, PT-derivatized beads have to be prepared fresh and quality controlled for each selection round. Since this procedure is quite time consuming, an alternative immobilization strategy involving a biotinylation of PT was evaluated.

6. *Semi-preparative biotinylation of PT*

A biotinylation reaction was performed by incubation of 0.4 mg (~3.65 nmole) reconstituted PT (1 µg/µl in CTW buffer) with 25 µg EZ-link-sulfo-NHS-LC-LC-biotin (PIERCE) in a final volume of 740 µl 50 mM HEPES, 150 mM NaCl, 0.2% Triton-X100. After an incubation period of 2 h on ice under permanent agitation the biotinylation reaction was quenched by addition of 74 µl 1M Tris/HCl pH 7.0. Subsequently, the protein was dialyzed against HEPES-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X100) at 4°C using a Slide-a-lyzer cassette (PIERCE, 3500 MWCO 0.5-3 ml) to remove the excess of biotinylation reagent. The biotinylated PT was removed from the dialysis cassette and stored in aliquots at –20°C.

7. *Quality control of biotinylated PT using a BIAcore instrument*

The quality of the biotinylation reaction was controlled by analysis of the interaction of biotinylated PT with a BIAcore streptavidin chip using BIAcore instrument (BIAcore 2000). It was also possible to detect the binding of asialofetuin to chip immobilized biotinylated PT (binding signal of ~ 400 RU to immobilized PT; unspecific binding of ~ 100 RU to the control cell).

F. Analysis of biotinylated PT for binding to streptavidin magnetic beads and to asialofetuin

1. Binding of biotinylated PT to streptavidin magnetic beads

20 µl streptavidin magnetic beads (Dynal) were incubated with 20 pmole of biotinylated PT in 1x HBS-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 1 mg/ml BSA, 100 µg/ml salmon sperm DNA, 0.025 % Triton-X100) for 1 h at RT, washed 3x with HEPES-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X100) and resuspended in 16 µl SDS-gel-loading buffer. 8 µl were analyzed by SDS-PAGE to confirm conjugation. In a negative control experiment under comparable conditions, free PT (not biotinylated) did not interact with streptavidin magnetic beads.

2. Binding of asialofetuin to bead immobilized biotinylated PT

20 µl streptavidin magnetic beads (Dynal) were incubated with 20 pmole of biotinylated PT in 1x HBS-buffer for 1 h at RT, washed 4x with HEPES-buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.025 % Triton X100). Subsequently, beads with immobilized biotinylated PT were incubated with 40 pmole asialofetuin in HEPES-buffer for 1 h at RT. After 4 washes with HEPES-buffer beads were resuspended in 16 µl SDS-gel-loading buffer. 8 µl were analyzed by SDS-PAGE to confirm binding. A simultaneous incubation of biotinylated PT and asialofetuin to the streptavidin magnetic beads instead of serial incubations resulted as well in binding of asialofetuin to biotinylated PT. In a comparable control experiment, it was determined that asialofetuin did not interact with the streptavidin magnetic beads non-specifically. Similar quality controls with biotinylated PT that has been stored for one week at –20°C showed no significant decrease in streptavidin and/or asialofetuin binding competence. Therefore, biotinylated PT was used as standard target in subsequent selections.

Example 2

Isolation of Peptides Selective for PT

The gurmarin PROfusion™ library and PT immobilized to magnetic beads were then contacted under strictly controlled stringency conditions. These conditions allow predominately those variants of the PROfusion™ library showing elevated affinity for PT to bind to the targets. After extensive washes that dilute unwanted, non-specific binding variants, the bound PROfusion™-molecules are eluted from the beads and are subjected to a new PROfusion™-formation cycle as shown in (**Figure 2**). By successive rounds of selection and

re-amplification along with a fine adaptation of stringency conditions a population of highly specific binding molecules to the given target is enriched (10). Subsequently the DNA-portion of this population is cloned into an *E. coli* plasmid vector to isolate individual variants that can be analyzed in detail by sequencing.

Six successive selection rounds against immobilized PT have been performed with the gurmarin PROfusion™-library. According to the perception described above, biotinylated PT immobilized to streptavidin beads has been used in these selections (**Table 1**). In selection round 4, a low background binding of the gurmarin pool to streptavidin beads has been observed which might indicate a starting enrichment of bead and/or streptavidin binding gurmarin variants. Therefore, in the following fifth selection round two individual selections were performed using biotin/streptavidin immobilized PT as target and epoxy bead coupled PT, respectively. In both selections, a clear background corrected enrichment of target binding was observed (**Table 1**). This trend has been confirmed in the sixth selection round using biotin/streptavidin immobilized PT, clearly indicating an accumulation of PT-binding variants (**Table 1**).

A. Cloning of selected gurmarin binder pools

The gurmarin DNA-pools resulting from selection rounds R4, R5 and R6 were cloned into the pCR®2.1-TOPO®-vector using the TOPO TA Cloning® kit (Invitrogen). The gurmarin DNA was ligated to the pCR®2.1-TOPO®-vector in different concentrations. For 6 µl reactions, 0.5 µl, 2 µl and 4 µl of the gurmarin pool DNA were used respectively. The ligation was performed according to the manufacturers instructions.

Two (2) µl of these ligations were transformed into 20 µl of the *E. coli* Top 10 F' competent cells (Invitrogen) and spread out on LB plates containing 50 µg/ml Kanamycin and 0.5 % Glucose. From each of these transformations 150 single colonies were picked to a masterplate containing 50 µg/ml Kanamycin and 0,5 % Glucose to repress T7 dependent protein expression and a second plate containing X-Gal and IPTG for a blue white screening. For each transformation, 96 of the colonies from the repressed masterplate corresponding to the white colonies from the blue white test were used to inoculate a 96 well LB agar plate and 500 µl liquid cultures (LB containing 50 µg/ml Kanamycin and 0,5 % Glucose). The 96 well agar plates were sent out for commercial sequencing service. The liquid cultures were mixed with 500 µl 40 % Glycerol, frozen in liquid nitrogen and stored at – 80 °C.

From each individual clone, plasmid DNA was prepared and subjected to an automated DNA-sequencing procedure using a M13-primer 5'- TGT AAA ACG ACG GCC

AGT-3'. As shown in **Figures 3-8**, a single gurmarin sequence variant begins to be significantly enriched in selection round 4 and represents > 90 % of all sequences after selection round 6. This clearly indicates that this variant probably binds with the highest affinity to PT. In addition to this most prominent sequence variant, a variety of other gurmarin sequences have been enriched that partially share common sequence motifs. This finding indicates that these other sequences show affinity towards PT as well.

B. PP26 affinity selection against immobilized PT

In parallel to the gurmarin selection six successive selection rounds against immobilized PT have been performed with the PP26 PROfusion™-library. Biotinylated PT immobilized to streptavidin beads has been used in these selections (**Table 2**). In selection round 4, a low background binding of the gurmarin pool to streptavidin beads has been observed which might indicate a starting enrichment of bead and/or streptavidin binding PP26 variants. Therefore, in the following fifth selection round two individual selections were performed using on the one hand biotin/streptavidin immobilized PT as target and on the other hand epoxy bead coupled PT. In both selections a clear background corrected enrichment of target binding have been detected (**Table 2**). This trend was confirmed in the sixth selection round using biotin/streptavidin immobilized PT, thus, clearly indicating an accumulation of PT-binding variants.

C. Cloning of selected PP26 binder pools

The PP26 DNA-pools resulting from selection rounds R4, R5 and R6 were cloned into the pCR®2.1-TOPO®-vector using the TOPO TA Cloning® kit (Invitrogen). The PP26 DNA was ligated to the pCR®2.1-TOPO®-vector in different concentrations. For 6 µl reactions 0,5 µl / 2 µl and 4 µl of the gurmarin pool DNA were used respectively. The ligation was performed according to the manufacturers instructions. 2 µl of these ligations were transformed into 20 µl of the *E. coli* Top 10 F' competent cells (Invitrogen) and spread out on LB plates containing 50 µg/ml Kanamycin and 0,5 % Glucose. From each of these transformations 150 single colonies were picked to a masterplate containing 50 µg/ml Kanamycin and 0,5 % Glucose to repress T7 dependant protein expression and a second plate containing X-Gal and IPTG for a blue white screening. For each Transformation 96 of the colonies from the repressed masterplate corresponding to the white colonies from the blue

white test were used to inoculate a 96 well LB agar plate and 500 µl liquid cultures (LB containing 50 µg/ml Kanamycin and 0,5 % Glucose). The 96 well agar plates were sent out for commercial sequencing service. The liquid cultures were mixed with 500 µl 40 % Glycerol, frozen in liquid nitrogen and stored at – 80 °C.

D. Sequencing of individual binder variants

From each individual clone plasmid DNA was prepared and subjected to an automated DNA-sequencing procedure using a M13-primer 5'- TGT AAA ACG ACG GCC AGT-3'. As shown in **Figures 9-14**, two main variants have been enriched during the selection rounds. Both variants share a common conserved sequence motif. This finding indicates that the side chains of the conserved amino acids putatively establish a direct interaction with a certain PT surface region. Furthermore, at least 4 additional variants have been enriched at lesser extent. Since these variants do not comprise the above mentioned conserved sequence motif it can be concluded that these variants potentially bind to different surface regions of PT.

E. Validation of selected PT-binding gurmarin- and PP26-variants

Since the selections were performed with PROfusion™-molecules – mRNA-peptide-fusions – it is necessary in the first step of the post selection analysis to check the free peptides for their ability to bind do the target. In the next step, those variants that establish their target binding through the peptide and not the nucleic acid portion are subjected to a specificity test in the presence of AP process fluids. By this measure, those variants should be identified that are most suitable to the AP process.

1. Test of free peptides for their binding capacity to PT

For a qualitative binding assay of free peptides of single enriched gurmarin- and PP26-binder variants the TNT T7 coupled Reticolocyte Lysat System (Promega #L5540) was used, as follows. DNA of single binder candidates was amplified by colony-PCR out of the glycerol stock of binder clones. To avoid mutations during PCR a proofreading polymerase (Pwo) was used. The PCR products were analyzed on a 2% agarose gel. 5.0 µl of PCR product were used as template for coupled *in vitro* transcription/translation reaction using the TNT system in a final volume of 53 µl according to the manufacturers instructions. Expressed binder candidates were subsequently purified by Ni-NTA chelat chromatography (QIAGEN). Radioactively labeled His-tag purified binder candidates (~40-70 fmol of each peptide) were incubated with biotinylated PT immobilized on streptavidin-magnetic beads for 1h at RT. The

beads were washed 3x with HBS-buffer and then resuspended in water and analyzed by liquid scintillation counting. In control experiments each candidate was incubated with streptavidin beads only (without PT). The best binder candidates of PP26 and gurmarin were identified (**Tables 3 and 4**, below) and were subjected to the following specificity test.

2. Specificity test of gurmarin and PP26 variants in the presence of process fluids

For a semi-quantitative binding and specificity assay of free gurmarin and PP26 peptides in the presence of Aventis Pasteur process fluids the peptides were first produced as PROfusion™, purified to homogeneity and than transferred to free peptides by an S1-nuclease digest. For amplification of a sufficient amount of DNA of the selected binder variants (10 Gurmarin clones and 7 PP26 clones) a PCR was performed using a PCR product from TNT expression as template. After 10 cycles of PCR (94°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec) the samples were analyzed on a 2% agarose gel. dsDNA (PCR product) was transcribed into RNA using the RiboMax Express *In vitro* transcription kit from Promega. After incubation for 45 min at 37°C, DNase I was added and the incubation at 37°C continued for an additional 15 minutes. This mixture was subjected to a phenol/chloroform extraction. Excess of NTPs was removed by NAP-5 gel filtration (Pharmacia). RNA was analyzed on a 6%-TBU-gel.

Purified RNA was annealed (85°C, 1 min cool down to 25°C at a ramp of 0.3°C/s) to a 1.5-fold excess of puromycin-oligonucleotide linker PEG2A18: 5'-psoralen-*UAG CGG AUG C* A_{18} $(PEG\text{-}9)_2$ CC puromycin (nucleotides shown in italics represent 2'-O-methyl-derivatives). The covalent coupling was performed by illumination for 15 min at RT (RT) with UV-light (365 nm). The reaction product was analyzed on 6%-TBU gel. Ligated RNA was translated using the rabbit reticulocyte lysate from Promega in the presence of 15 µCi ^{35}S-methionine (1000 Ci/mmole). After a 30 min incubation at 30°C, KCl and $MgCl_2$ were added to a final concentration of 530-mM and 150 mM respectively and a sample was analyzed on 4-20% Tris/glycine-SDS-PAGE. mRNA-protein fusions (PROfusions ™) were isolated by incubation with oligo dT magnetic beads (Miltenyi) in incubation buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM NaCl and 0.25 % Triton X-100) for 5 min at 4°C. PROfusion™ molecules were isolated by filtration through MiniMACS-columns (Miltenyi), washing with incubation buffer and elution with water. A sample was analyzed on 4-20% Tris/glycine-SDS-PAGE.

To remove the mRNA part of the mRNA-protein fusions the oligo dT purified molecules were digested with S1-Nuclease (S1-Nuclease cleaves the DNA-part of the Puromycin linker) according to the manufacturers instructions. Samples of the PROfusion

molecules before and after S1-digest were analyzed on 4-12 % Bis/Tris SDS-PAGE. Streptavidin beads (M280 Dynal) were washed in HBS and incubated o/n at 4°C. Biotinylated PT (900 pmol) was incubated with 900 µl Strepbeads (preblocked in HBS buffer) for 1h at RT. After immobilization of PT, the beads were blocked with biotin (2 mM biotin in HBS) for 1 min and immediately washed 4x with HBS buffer to remove any traces of biotin. Control beads (without PT) were blocked with biotin in the same way.

For binding analysis of the selected peptides several parallel reactions were set up, as follows: negative control only with biotin blocked Streptavidin beads; positive control with PT immobilized on Streptavidin beads; background control with biotin blocked beads in combination with ¼ volume Aventis Pasteur sample-solution C (flow through 1. AF column); mix of PT in combination with ¼ volume of sample-solution C; background control with biotin blocked beads in combination with ¼ volume of Aventis Pasteur sample-solution E (culture medium); mix of PT in combination with ¼ volume of sample-solution E; reactions 3-6 were performed to investigate the capacity of the selected peptides to bind PT specifically in the presence of samples provided by Aventis Pasteur. Binding was done for 1h at RT in the presence of a protease inhibitor mix (complete mini™ ROCHE), to avoid degradation of the peptides. After washing with HBS solution the beads were analyzed by scintillation counting.

As shown in **Table 3**, three (#9, 10 19) of the ten tested gurmarin variants show a target binding to PT that is not influenced by any of the AP process fluids. These variants are the most promising candidates for affinity chromatographic applications within the AP process.

As shown in **Table 4** three (#5, 6 9) of the seven tested PP26 variants show target binding to PT that is not reduced by the AP process fluids. These variants are the most promising candidates for further affinity chromatographic applications within the AP process.

Table 3

Post selection analysis of gurmarin-variants*

#	seq #	peptide sequence	test 1	test 2
1	194227	MHHHHHHSGSSSGSGCVKKDELCAGSVGHCCEPLECLRRFLNLRWCGSGSSGSS	-	n.d.
2	194238	MHHHHHHSGSSSGSGCVKKDELCIVMRAPCCEPLECLRRYMLKHMCGSGSSGSS	-	n.d.
3	194239	MHHHHHHSGSSSGSGCVKKDELCKAFRYSCCEPLECLRKWLKARFCGSGSSGSS	-	n.d.
4	194251	MHHHHHHSGSSSGSGCVKKDELCLRSSIDCCEPLECLYKWMQRRLCGSGSSGSS	-	n.d.
5	194210	MHHHHHHSGSSSGSGCVKKDELCWPRRHKCCEPLECLLEMLERKRCGSGSSGSS	-	n.d.
6	194261	MHHHHHHSGSSSGSGCVKKDELCMSMACVCCEPLECKYHGYFWLCGSGSSGSS	-	n.d.
7	194214	MHHHHHHSGSSSGSGCVKKDELCAVWFDVCCEPLECTYQSGYYWLCGSGSSGSS	-	n.d.
8	194226	MHHHHHHSGSSSGSGCVKKDELCEPWYWRCCEPLECVYTSGYYYSCGSGSSGSS	-	n.d.
9	194259	MHHHHHHSGSSSGSGCVKKDELC*ARWDLV*CCEPLEC*IYTSELYAT*CGSGSSGSS	√	√
12	194297	MHHHHHHSGSSSGSGCVKKDELCVFYFPNCCEPLECRWVNDNYGWCGSGSSGSS	√	-
13	194330	MHHHHHHSGSSSGSGCVKKDELCMSMACVCCEPLECKYHGYFWLCGSGSSGSS	√	-
14	194479	MHHHHHHSGSSSGSGCVKKDELCTTASKSCCEPLECKWTNEHFGTCGSGSSGSS	√	-

15	194511	MHHHHHHSGSSSGSGCVKKDELCSQSVPMCCEPLECKWFNENYGICGSGSSGSS	√	-
16	194533	MHHHHHHSGSSSGSGCVKKDELCARWDLVCCEPLECIYTSELYATCGSGSSGSS	√	-
17	194486	MHHHHHHSGSSSGSGCVKKDELCARWDLVCCEPLECLGHGLGYAYCGSGSSGSS	-	n.d.
18	194668	MHHHHHHSGSSSGSGCVKKDELCMWSREVCCEPLECYYTGWYWACGSGSSGSS	-	-
10	194264	MHHHHHHSGSSSGSGCVKKDELC*ELAVDE*CCEPLEC*FQMGHGFKR*CGSGSSGSS	√	√
19	194737	MHHHHHHSGSSSGSGCVKKDELC*ELAVDE*CCEPLEC*TKGDLGFRK*CGSGSSGSS	√	√
20	194716	MHHHHHHSGSSSGSGCVKKDELCELAIDVCCEPLECLGHGLGYAYCGSGSSGSS	√	n.d.
21	194720	MHHHHHHSGSSSGSGCVKKDELCELAIDVCCEPLECLGHGLGYAYCGSGSSGSS	-	-
11	194328	MHHHHHHSGSSSGSGCVKKDELCNWVTPMRCEPLECLGHGLGYAYCGSGSSGSS	√	n.d.

*Test 1 represents the target binding ability of free peptides (0) and test 2 represents the binding specificity of variants in the presence of AP process fluids (0). Variants that are positive in both assays are 9, 10, and 19.

Table 4

Post selection analysis of PP26-variants*

#	seq #	peptide sequence	test 1	test 2
1	197569	MGRGSHHHHHHARSDWELSPPHVAITTRHLINCTDGPLLRDANAPKASAI	-	n.d.
2	197536	MGRGSHHHHHHARSLNGESTSNILTTSRKVTEWTGYTASVDANAPKASAI	-	n.d.
3	197611	MGRGSHHHHHHARSQVTWHHLADTVTTKNRKCTDSYIGWNXANAPKASAI	-	n.d.
4	197530	MGRGSHHHHHHARSIIVIHNAIQTHTPHQVSIWCPPKHNRDANAPKASAI	-	n.d.
5	197557	MGRGSHHHHHHARSSHCRHRNCHTITRGNMRIETPNNIRKDANAPKASAI	√	√
6	197596	MGRGSHHHHHHARSTMNTNRMDIQRLMTNHVKRDSSPGSIDANAPKASAI	√	√
7	197552	MGRGSHHHHHHARSLSALRRTERTWNTIHQGHHLEWYPPADANAPKASAI	-	n.d.
8	197541	MGRGSHHHHHHARSWTSMQGETLWRTDRLATTKTSMSHPPDANAPKASAI	-	n.d.
9	197588	MGRGSHHHHHHARSNVIPLNEVWYDTGWDRPHRSRLSIDDDANAPKASAI	√	√
10	197635	MGRGSHHHHHHARSCLATRNGFV.MNTDRGTYVKRPTVLQDANAPKASAI	√	-
11	197797	MGRGSHHHHHHARSWGLSGTQTWKITKLATRLHHPEFETNDANAPKASAI	-	n.d.
12	197888	MGRGSHHHHHHARSWRWHNWGLSDTVASHPDASNSLNMMYDANAPKASAN	-	n.d.
13	197897	MGRGSHHHHHHLDLWGPPSGSPRTRSTTGTSTTSSPSTPGTLTLRRHPH	-	n.d.
14	197825	MGRGSHHHHHHARSWQPEVKMSSLVDTSQTVGAAVETRTTDANAPKASA	√	-
15	198000	MGRGSHHHHHHARSWRDTRKLHMRHYFPLAIDSYWDHTLRDANAPKASAI	√	-
16	197983	MGRGSHHHHHHARSWTSMQGETLWRTDRLATTKTSMSHPPDANAPKASAI	-	n.d.
17	197998	MGRGSHHHHHHHARSPLWYHYNCWDTICLADWLKDRPHGVYDANAPKASA	-	n.d.
18	197947	MGRGSHHHHHHARSVGTTIRIAQDTEHYRNVYHKLSQYSRDANAPKASAI	√	-
19	197954	MGRGSHHHHHHARSVGTTIRIAQDTEHYRNVYHKLSQYSRDANAPKASAI	-	n.d.
20	197971	MGRGSHHHHHHARSNVIPLNEVWYDTGWDRPHRSRLSIDDDANAPKASAI	-	n.d.

*Test 1 represents the target binding ability of free peptides (0) and test 2 represents the binding specificity of variants in the presence of AP process fluids (0). Variants that are positive in both assays are 5, 6 and 9.

F. Peptide production by chemical synthesis

Eight different peptides were produced by chemical synthesis in form of N-terminal biotinylated peptides. The Biotin group was spaced via a short hydrophilic linker (PEG2 = 8-Amino-3,6-dioxaoctanoic acid). Two of these 8 peptides (PP26-5c and gumarin-9c) were additional synthesized in form of C-terminal tagged biotinylated peptides (via an additional C-terminal Lysine). The peptides were automatically synthesized using the Fmoc/But strategy according to Sheppard, purified by HPLC and subsequently lyophilized. The quality of all purified peptides was confirmed by mass spectroscopy. The target quantity of each peptide synthesis was5 mg purified peptide. An overview about yield and purity of the synthetic peptides after purification is given in **Table 5.**

Table 5

Peptide Synthesis of Pertussis Toxin Binding Peptides*

Selection	Clone	Seq #	Sequence	Purity (%)	Yield (mg)
pp26	5 c	197557	RSSHCRHRNCHTITRGNMRIETPNNIRKDAK	90 - 95	7,7
pp26	5 n	197557	RSSHCRHRNCHTITRGNMRIETPNNIRKDA	90 - 95	7,6
pp26	6 n	197596	RSTMNTNRMDIQRLMTNHVKRDSSPGSIDA	90 - 95	6,3
pp26	9 n	197588	RSNVIPLNEVWYDTGWDRPHRSRLSIDDDA	90 - 95	5,8
pp26	15 n	198000	RSWRDTRKLHMRHYFPLAIDSYWDHTLRDA	90 - 95	4,8
gurmarin	9 c	194259	SGCVKKDELCARWDLVCCEPLECIYTSELYATCGK	70	1,0
gurmarin	9 n	194259	SGCVKKDELCARWDLVCCEPLECIYTSELYATCG	80 - 90	4,0
gurmarin	10 n	194264	SGCVKKDELCELAVDECCEPLECFQMGHGFKRCG	90 - 95	4,9
gurmarin	15 n	194511	SGCVKKDELCSQSVPMCCEPLECKWFNENYGICGS	90 - 95	6,3
gurmarin	19 n	194737	SGCVKKDELCELAIDECCEPLECTKGDLGFRKCG	90 - 95	6,7

*Abbreviation c in the clone name indicates C-terminal biotinylated peptides, abbreviation n indicates N-terminal biotinylated peptides.

All pp26 peptides were dissolved in 100 mM HEPES, pH 7.4, 200 mM NaCl with a final concentration of 100 µM. All gurmarin peptides were dissolved in 100 mM HEPES, pH 7.4, 200 mM NaCl, 2 mM GSH, 1 mM GSSG with a final concentration of 100 µM and subsequently incubated under nitrogen for at least 48 hours to allow structural folding.

G. Peptide production by bacterial expression

The peptides which were identified as binders to the pertussis toxin were subcloned in frame to glutathione-S-transferase (GST) and expressed bacterially. The GST-tag enhances the solubility and allows purification using Glutathione Sepharose. An enginered protease cleavage site recognized by the specific PreScission™ protease allows removal of the GST-tag releasing the peptide. The PreScission™ protease itself is a fusion protein of GST and human rhinovirus (HRV) type 14 3C protease and specifically recognizes the sequence Leu-Phe-Gln*Gly-Pro cleaving between the Gln and Gly residues. After the cleavage the uncleaved product as well as the protease can be removed from the cleavage reactions using Glutathione Sepharose.

H. Construction of expression vectors

1. Construction of GST fusions for pp26-variants

As template for PCR served the pCR2.1 vector containing the sequences of the identified pp26 binders to PT. The products obtained in a PCR using the oligonucleotides #467 (5′-CATGCCATGGGACGTGGCTCACATCATC-3′) and #468 (5′-phosphate-GGGTTAAATAGCGGATGCCTTCGGAGCGTTAGCGTC-3′) with *Pwo* DNA polymerase (Roche) were digested with *Nco*I (New England Biolobs). A modified vector (pGEX6P

(Amersham/Pharmacia) containing an additional *Nco*I site) was digested with *NcoI/Sma*I (New England Biolobs) and the PCR product was directionally cloned into the *NcoI/Sma*I site of this vector. After transformation in TOP10 (Invitrogen) positive clones were identified by colony PCR and verified by sequencing.

5

2. Construction of GST fusions for gurmarin-variants

As template for PCR served the pCR2.1 vector containing the sequences of the identified gurmarin binders to PT. The products obtained in a PCR using the oligonucleotides #464 (5'-GGAGATCTCATATGCACCATCACCATCACCATAGTGGC-3') and #465 (5'-phosphate-GGGTTAAATAGCGGATGCTACTAGGC-3') with *Pwo* DNA polymerase (Roche) were digested with *Nde*I (New England Biolobs). A modified vector (pGEX6P (Amersham/Pharmacia) containing an additional *Nde*I site) was digested with *NdeI/Sma*I (New England Biolobs)and the PCR product was directionally ligated into the *NdeI/Sma*I site of this vector. After transformation in TOP10 (Invitrogen) positive clones were identified by colony PCR and verified by sequencing (**Table 6**).

Table 6
Vectors used for bacterial expression

Plasmid number	**pp26**
pS840	pGEX6P-$(His)_6$-pp26 K5
pS850	pGEX6P-$(His)_6$- pp26K6
pS841	pGEX6P-$(His)_6$- pp26K9
pS842	pGEX6P-$(His)_6$- pp26K15
	gurmarin
pS836	pGEX6P-$(His)_6$-gurmarin K9
pS837	pGEX6P-$(His)_6$-gurmarin K10
pS838	pGEX6P-$(His)_6$-gurmarin K15
pS839	pGEX6P-$(His)_6$-gurmarin K19

3. Expression and purification of GST-pp26 fusions

The bacterial strain Rosetta (DE3) pLysS (Novagen) was transformed with plasmid DNA (see Table). The transfomands of the pp26 variants were grown at 37°C 250rpm to an OD_{600} of ~ 0.5 and induced by the addition of 1mM IPTG for 4h. In case of gurmarin-GST-fusions the induction was performed for 2.5 hours using 0.33 mM IPTG. After harvesting the bacterials, cells were resuspended in PBS-KMT (10 mM Na phosphate, pH 7.5,130 mM NaCl, 3 mM KCl, 1 mM MgCl, 0.1% Tween-20), containing 1 mM 2-Mercaptoethanol, protease inhibitors and 1 mM Lysozyme, incubated for 30 min at RT and disrupted by sonification. The soluble supernatant after centrifugation was transferred to GSH sepharose column for purification. After washing the column with 10 column volumes of 20 mM Hepes,

pH 7.5, 150 mM NaCl the GST fusion protein was eluted with 20mM GSH and analyzed on a SDS gel to confirm expression.

4. ***Peptide generation by removal of GST tag by cleavage with PreScission™ Protease***

An example for PreScission™ cleavage of one peptide from the GST-peptide fusion is shown below. The GST-tag was removed by incubation with PreScission™ Protease (Amersham Pharmacia): 2.5 mg of fusion protein was incubated with 160U PreScission™ and digested for 16 hours at 5°C on the sealed GSTrap FF column containing the bound GST fusion protein. After the overnight incubation a second GSTrap FF column was connected to remove the GST-tagged protease PreScission™. The sample was applied with a flow rate of 0.2 ml/min, the flow through was collected in small aliquot samples and analyzed by SDS gel electrophoresis and the amount of peptide was calculated by OD_{280} measurement (ca. 700 µg).

Example 3

Affinity Purification of PT

A. Analysis of fermentation supernatant on denaturing gels

Two process fluids were considered as potential starting material for affinity chromatography process:

Sample A Concentrated culture filtrate containing 10-50 µg/ml (~0.09-0.45 µM) crude PT, fermentation supernatant

Sample B Absorption chromatography supernatant containing 9-45 µg/ml (~0.08-0.4 µM) crude PT

To visualize the complexity of these process fluids, both samples were analyzed by denaturating polyacrylamid gelelectrophoresis. Mainly high molecular weight components of sample A are removed by the absorption chromatography (sample B).

B. Immobilization of synthetic biotinylated core peptides to Streptavidin sepharose and verification of binding to purified PT

1. Peptide immobilization to Streptavidin sepharose

For binding of biotinylated peptides to streptavidin sepharose (Amersham High Performance 71-5004-40), 200 µl of 50% slurry of pre-washed streptavidin sepharose were incubated with 1 nmol peptide (10 µl of 100 µM peptide solution) in 1 ml HEPES buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.025% TritonX-100) at 4°C. Under the applied

conditions the high binding capacity of streptavidin sepharose should allow immobilization of 100% of the biotinylated peptide (10 pmol peptide per µl packed sepharose).

2. Binding of purified PT to immobilized peptides

200 µl of sepharose loaded with peptides (10%ige slurry, containing immobilized ~200 pmol peptide) were transferred to a Mobicol column (MoBiTec, 10 µm filter) and the supernatant was removed by centrifugation for 1 min at 2000 rpm. After 4 washes with HEPES buffer, the sepharose was resuspended in 200 µl HEPES buffer containing 100 pmol purified Pertussis Toxin and incubated on a rotating wheel for 1 hour at room temperature. The unbound fraction was separated by centrifugation (supernatant after binding; applied to gel analysis). Subsequently the peptide-streptavidin sepharose was washed three-times with cold HEPES buffer (each 200 µl) and resuspended in 20 µl loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% Glycerol, 0.005% Bromphenol Blue) to elute bound PT. After 5 min incubation at 95°C the loading buffer was collected by centrifugation and subsequently used for gel analysis (**Figure 15**). As a control streptavidin sepharose without peptide was contacted with PT under identical conditions. Under the applied conditions the Pertussis toxin peptide binder clones pp26 5n, 5c, 9n, 15n and the gurmarin clones 10n, 19n, 15n, 9n show a clear binding to purified PT. All positive binder candidates were able to bind the intact hexameric PT.

C. Immobilization of synthetic biotinylated core peptides to Streptavidin sepharose and verification of binding to PT out of the fermentation supernatant.

Peptide immobilization to streptavidin sepharose and binding analysis to PT out of fermentation supernatants was performed as described in chapter 0 with the exception that the peptide streptavidin sepharoses were incubated with 200 µl Sample A (fermentation supernatant) or with 200 µl Sample B (absorption chromatography supernatant column, see chapter 0) and were subsequently washed 4-times with HEPES buffer at RT. The results of the binding analysis is presented in **Figure 16**. Under the applied conditions the Pertussis toxin pp26 binder clones 9n, 15n and the gurmarin clones 9n, 15n were able to bind very efficiently the intact PT hexamer out of the fermentation supernatants Sample A and Sample B. Note that under the applied conditions the pp26 binder clone 5 and the gurmarin binder clones 10 and 19 might bind PT with lower affinity. Although the PT binding to these peptides out of Sample A and Sample B were not detected under the conditions, these binders might be still qualified for application as ligand in an affinity chromatography column (a

column allows retention of PT by rebinding effects and therefore would minimize the k_{off} problematic).

D. Thermodynamic data on immobilized peptides

For estimation of peptides binding capacity 20 pmol of sepharose-immobilized peptides were incubated with an excess of 100 pmol PT in a volume of 200 µl HEPES buffer (corresponds to 500 nM PT). After washing, the fraction of peptide-streptavidin sepharose bound PT was quantified by gel analysis. This allows directly to calculate the fraction of binding active peptide under the applied conditions (assuming the PT/peptide binding ratio is 1:1). Under the assumption that a concentration of 500 nM PT is high enough to reach B_{max} for all peptides. The results of the analysis are shown in **Table 7**. The values presented therein are estimations for the expectable binding capacities of the peptides. An exact evaluation of binding capacity (B_{max}) and dissociation constant (K_D) of the most suitable binder may also be performed.

Table 7

Overview about fraction of binding active peptides under the applied experimental conditions

Peptide name	pp26 5n	pp26 9n	pp26 15n	gurm 9n	gurm 10n	gurm 15n	gurm 19n
Fraction of binding active peptide	>5% [1]	>50 %	>12,5 %	>12,5 %	>5% [1]	>50 %	>5% [1]

[1] Calculation difficult because signals were near the detection limit

E. Analysis of the stability of the purified Pertussis toxin hexamer under defined buffer conditions (pH, salt concentration, detergents), using acceptable quality grade raw materials versus Health Authorities requirements

The Pertussis toxin hexamer stability was tested under a broad range of pH and salt conditions on a BIAcore 2000 instrument. For this purpose ~2000 RU of biotinylated PT were loaded on a streptavidin chip. Subsequently different buffers were applied to the chip immobilized PT for 2 min with a flow rate of 30 µl/min. After the end of each buffer injection the chip was equilibrated with HBS/EP running buffer (0,01 M HEPES pH 7.4, 0.15 M NaCl 3 mM EDTA 0.005% polysorbate 20 (v/v), at least 2 min with a flowrate of 30 µl/min). The difference of the measured RU signal before and after buffer injection correlates to the reduction of PT hexamer on the chip. This reduction was interpreted as loss of stability of PT hexamer under the aplied buffer conditions.

The analyzed pH range was between pH 2 and 10.5 using the following buffers: 10 mM glycin buffer (BIAcore, pH 2; 2.5; 3), 10 mM acetate buffer (BIAcore, pH 4; 4.5; 5; 5.5), 50 mM Tris/HCl (pH 8.5) and 100 mM carbonate buffer (pH 9.6 and 10.5). BIAcore sensograms demonstrating the influence of the pH on the PT hexamer stability were generated, and the results of the BIAcore analysis are summarized in **Table 8**. Under the applied conditions, Pertussis toxin hexamer was shown to be stable over a broad pH range between pH 2.5 – 10.5.

Table 8

Pertussis toxin hexamer stability under different pH conditions.

pH	2	2.5	3	4	4.5	5	5.5	8.5	9.6	10.5
PT hexamer stability (%)	93	98	100	100	100	100	100	100	98	95

The influence of different salt conditions on the PT hexamer stability were investigated in comparable experiments on the BIAcore 2000 instrument for NaCl, KCl and $MgCl_2$ at pH 5.0 (10 mM acetate buffer) and pH 8.5 (50 mM Tris/HCl) respectively. An overview about PT hexamer stability under the applied salt conditions is shown in **Table 9**. The hexamer was stable in buffer (at pH 5 and 8.5) containing up to 2.5 M NaCl or up to 2 M KCl. In case of $MgCl_2$ the PT hexamer was stable in a buffer containing up to 2 M $MgCl_2$ at pH 8.5.

Table 9

Pertussis toxin hexamer stability under different salt conditions

PT hexamer stability	pH 5	pH 8.5
NaCl	**0 – 2.5 M** stable	**0 – 2.5 M** stable
KCl	**0 – 2.0 M** stable	**0 – 2.0 M** stable
$MgCl_2$	**Nd**	**0 – 2.0 M** stable

F. Establish defined wash and elution conditions allowing a specific affinity purification of PT out of fermentation supernatant (pH, salt concentration, detergents).

After the determination under which conditions the Pertussis toxin hexamer is stable, the next step was to investigate the wash and elution conditions for the bound Pertussis toxin to the immobilized peptides pp26 clone 9 and 15 and gurmarin clone 9 and 15.

1. Evaluation of PT/ peptide stability using the BIAcore 2000 instrument

The stability of PT/peptide complexes were investigated using the BIAcore 2000 instrument under different pH and salt conditions that were shown before not to interfere with the PT hexamer stability. 500 – 1000 RU of the synthetic peptides were immobilized on BIAcore streptavidin chips. To allow binding of PT to the immobilized peptides, 20 nM purified PT in HEPES buffer was injected for 1 minute. After equilibration with HBS/EP running buffer (0,01 M HEPES pH 7.4, 0.15 M NaCl 3 mM EDTA 0.005% polysorbate 20 (v/v)) the PT/peptide complexes were washed by injection of

(a) 100 mM carbonate buffer at pH 10.5 and 9.5
(b) 10 mM acetate buffer at pH 5.5, 5.0, 4.5, and 4.0
(c) 10 mM glycin buffer at pH 3.0 and 2.5
(d) 0.5, 1.0, 1.5, 2.0 M NaCl in 10 mM acetate buffer buffer, pH 6.0,
(e) 0.5, 1.0, 1.5, 2.0 M NaCl in 50 mM Tris/HCl buffer, pH 8.5,
(f) 0.5, 1.0, 1.5, 2.0 M KCl in 10 mM acetate buffer, pH 6.0,
(g) 0.5, 1.0, 1.5, 2.0 M KCl in 50 mM Tris/HCl buffer, pH 8.5,
(h) 0.5, 1.0, 1.5, 2.0 M NaCl in 10 mM acetate buffer, pH 6.0,
(i) 0.5, 1.0, 1.5, 2.0 M NaCl in 50 mM Tris/HCl buffer, pH 8.5.

After the end of each buffer injection, the chip was equilibrated with HBS/EP running buffer. The loss of PT hexamer on the chip under the applied buffer conditions (difference of measured RU signal before and after buffer injection) reflects the PT/peptide complex stability. An overview about the pH range stability and salt stability of all PT/peptide complexes is summarised in **Table 10**. All of the PT/peptide complexes were completely destabilized in the presence of 100 mM carbonate, pH 10.5 as well as 10 mM glycin, pH 2.5. For gurmarin peptide 9, buffers containing 2.5 M NaCl or at least 0.5 M $MgCl_2$ interfere with PT/peptide complex stability. PT complexes with gurmarin peptide 15 were additionally destabilized in the presence of at least 1.5 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5).

Table 10

Effect of different pH and salt conditions on the stability of the PT/peptide complexes

	pp26 peptide 9		*pp26 peptide 15*		*gurmarin peptide 9*	*gurmarin peptide 15*	
pH range stability of the complex	3 - 9 stable, instable at pH 2.5 or 10.5		3 – 9 stable, instable at pH 2.5 or 10.5		3 – 9 stable, instable at pH 2.5 or 10.5	3 - 9 stable, instable at pH 2.5 or 10.5	
	pH 6	*pH 8,5*	*pH 6*	*pH 8,5*	*pH 8,5*	*pH 6*	*pH 8,5*
NaCl stability of the complex	2 M stable	2 M stable	2 M stable	2 M stable	strong sensitive to salt	sensitive to salt	
KCl stability of the complex	2 M stable	2 M stable	2 M stable	2 M stable	strong sensitive to salt	sensitive to salt	
$MgCl_2$ stability of the complex	stable	Sensitive from 125 mM, complete elution ≥ 2 M	stable up to 2 M	Elution ≥ 1.5, but not complete	Elution ≥ 0.5 M	Elution ≥ 1 M, complete elution ≥ 2 M	Elution ≥ 1 M, complete elution ≥ 2 M

2. Evaluation of wash conditions for purification of PT on peptide streptavidin sepharose

Wash conditions were tried to apply close to the established conditions for pertussis toxin purification process on asialofetuin (washing with 50 mM Tris/HCl, pH 7.5, with or without 1 M NaCl). The Pertussis toxin purification protocol was optimized for the peptides pp26 clone 9 and 15 and gurmarin clone 9 and 15. 200 pmol of each peptide immobilized on 20 µl sepharose were incubated with 100 µl 50 mM Tris/HCl, pH 7.5 and 100 µl sample A or sample B to allow binding of PT. Subsequently the peptide sepharose with bound PT fraction was washed under 3 different conditions, as shown below:

(a) 3 times with 200 µl 50 mM Tris/HCl, pH 7.5;

(b) 3 times with 200 µl 50 mM acetate pH 6.0; and,

(c) 6 times with 200 µl 50 mM acetate pH 6.0.

After washing remaining material was eluted from the sepharose with 20 µl loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% Glycerol, 0.005% Bromphenol Blue). All elutions were subsequently analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining (**Figure 17**).

Washing with 50 mM acetate, pH 6.0, is more stringent and reduces the back ground of high molecular weight impurities more efficient than washing with 50 mM Tris/HCl, pH

7.5. But under these washing conditions the PT/peptide complexes are less stable, especially in case of the gurmarin peptide 9 and repeated washes with 50 mM acetate, pH 6.0 (6 washes). In contrast to 50 mM Tris/HCl, pH 7.5, the loss of peptide immobilized PT was more dramatic when washing with 50 mM acetate, pH 6.0 was repeated 10 to 20 times (as an example shown for pp26 peptide 9 in **Figure 18**).

3. *Evaluation of elution conditions for purification of PT on peptide streptavidin sepharose*

Elution of PT from peptide sepharose was tested under conditions that are compatible with hexamer stability.

a. Elution by $MgCl_2$

As shown above by BIAcore 2000 measurements all PT/peptide complexes were sensitive against 2 M $MgCl_2$, conditions that were shown not to be critical for PT hexamer stability. The elution efficiencies of defined $MgCl_2$ concentrations were evaluated for PT that was bound on streptavidin sepharose via one of the four immobilized synthetic peptides. 400 pmol of each peptide immobilized on 20 µl sepharose were incubated with 100 µl 50 mM Tris/HCl, pH 7.5 and 100 µl sample A to allow binding of PT. After 4 washes with 50 mM Tris/HCl, pH 7.5 (200 µl each), the bound fraction of PT was eluted using 3 consecutive 20 µl volumes of

(a) 0.2 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5, or
(b) 0.5 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5, or
(c) 1.0 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5, or
(d) 1.5 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5, or
(e) 2.0 M $MgCl_2$ in 50 mM Tris/HCl, pH 8.5.

Remaining material was afterwards eluted from the peptide streptavidin sepharose with 20 µl loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% Glycerol, 0.005% Bromphenol Blue). All elutions were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining (**Figure 19**). As shown in the experiment elution with $MgCl_2$ was more efficient for the gurmarin peptides than for the pp26 peptides although a substantial amount of PT still remained on the peptide streptavidin sepharose.

b. Elution by pH-shift

The BIAcore measurements revealed that PT was elutable from all peptides with acidic (pH of 2.5) or basic (pH of 10.5) buffer conditions that were not critical for PT hexamer

stability (50 mM glycin, pH 2.5 more gentle for PT hexamer stability than 100 mM carbonat buffer, pH 10.5, see xxx). 200 pmol of each peptide immobilized on 20 µl sepharose were incubated with 100 µl 50 mM Tris/HCl, pH 7.5 and 100 µl sample A to allow binding of PT. After 4 washes with 50 mM Tris/HCl, pH 7.5 (200 µl each), PT was eluted from the peptide streptavidin sepharose by 3 consecutive 40 µl elutions with 50 mM glycin, pH 2.5, or 100 mM carbonate buffer, pH 10.5. Remaining material was subsequently eluted from the peptide streptavidin sepharose with 20 µl loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% Glycerol, 0.005% Bromphenol Blue). All elutions were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining (**Figure 20**). Nearly all of PT was elutable from the peptide streptavidin sepharose using 50 mM glycine, pH 2.5 as well as using 100 mM carbonate buffer, pH 10.5.

4. Apply optimized conditions for small scale purification scheme, confirm binding capacity

a. Purification of PT from Sample B under optimized wash and elution conditions (4 µl column)

Optimized wash and elution conditions were combined to allow the purification of PT on peptide streptavidin sepharoses out of Sample B. To reduce unspecific binding of PT the optimal peptide/streptavidin sepharose ratio was titrated for each peptide before. Subsequently the Sample B/peptide streptavidin sepharose ratio was optimized in respect to high recovery of PT per expectable high (moderate) input of peptide. These conditions were applied to the following small scale column purifications.

For pp26 peptide 9 and gurmarin peptide 15, the immobilization to streptavidin sepharose was performed by incubation of 16 µl streptavidin sepharose with 1600 pmol peptide. In case of pp26 peptide15, 16 µl streptavidin sepharose was incubated with 6000 pmol peptide (pp26/15 binds with lower efficiency to the streptavidin sepharose, might be explainable by incomplete peptide biotinylation). For gurmarin peptide 9, 8000 pmol were immobilized on 80 µl streptavidin sepharose. Subsequently the washed peptide streptavidin sepharoses were equally subdivided and transferred to 4 Mobilcom columns (with 10 µM filters).

Each column (containing 4 µl sepharose with 400 pmol peptide for pp26/9 and gur/15; 4 µl with undefined amount bound peptide pp26/15; 20 µl with 2000 pmol peptide for gur/9) was incubated with 400 µl Sample B (adjusted to pH 7.0 – 7.5 by addition of HCl) to allow binding of PT. After 5 washes with 50 mM Tris/HCl, pH 7.5 (each 100 µl), PT was eluted

from the peptide streptavidin sepharose by consecutive elutions (3 elutions for pp26/9 and gur/15; 4 elutions for pp26/15 and gur/9), as follows:

(a) with 50 mM ~~glycin~~ glycine, pH 2.5 (each 20 µl) in case of column 1, or

(b) with 100 mM carbonate buffer, pH 10.5 (each 20 µl) in case of column 2, or

(c) with 2 M $MgCl_2$ in 50 mM Tris, pH 8.5 (each 20 µl) in case of column 3.

Remaining material on column 1 – 3 as well on column 4 was subsequently eluted from the peptide streptavidin sepharoses by elution with 20 µl loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% Glycerol, 0.005% Bromphenol Blue). All elutions were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining (**Figures 21, 22**). To calculate the yield of PT after purification on the peptide streptavidin sepharoses the pooled elutions 1 – 3 were analyzed by PAGE and silver staining and compared to defined amounts of purified PT separated on the same gel allowing an estimation (**Figures 21B and 22B**). Based on the gel estimation, the yield of purified PT was calculated as shown in **Table 11**.

Table 11

Calculation of the Pertussis Toxin yield after small scale column purification from sample B with pp26 peptide 9 or gurmarin peptide 15 as affinity ligands

Peptide	Elution with	Estimation from figure 13B and 14B	Total yield of PT	Yield relative to input PT in sample B*	Yield relative to the amount of sepharose bound peptide
pp26 peptide 9	Glycin pH 2.5	2 pmol PT in 3/120 of pooled elutions	80 pmol	> 48%	20%
	Carbonat pH 10.5 elution	2 pmol PT in 3/120 of pooled elutions	80 pmol	> 48%	20%
	$MgCl_2$	1 pmol PT in 6/120 of pooled elutions	20 pmol	> 12%	5%
gurmarin peptide 15	Glycin pH 2.5	2 pmol PT in 3/120 of pooled elutions	80 pmol	> 48%	20%
	Carbonat pH 10.5 elution	2 pmol PT in 3/120 of pooled elutions	80 pmol	> 48%	20%
	$MgCl_2$	1 pmol PT in 3/120 of pooled elutions	40 pmol	> 24%	10%

* according to the documentation related to PT, the expected PT concentration of sample B is 9 – 45 µg/ml, corresponding to 0.8 – 0.41 pmol/µl. Calculation was performed as following:
400 µl of sample B x 0.41 pmol/µl = 164 pmol input PT

b. Determination of PT yield during affinity purification using varying peptide densities on streptavidin sepharose

The PT binding to peptide streptavidin sepharose was investigated in dependence of varying concentration of peptide immobilized on the streptavidin sepharose as affinity ligand. For immobilization 1 µl volume of streptavidin sepharose was incubated with increasing amounts of peptide pp26/9 or gurmarin/15 (100, 200, 300, 400, 500, 1000 pmol peptide). Unbound fractions of peptides were removed from the sepharose by 3 washes with 50 mM Tris/HCl, pH 7.5 (on column). Subsequently each peptide straptavidin matrix was incubated with 600 µl Sample A to allow binding of PT. After 80 min each matrix was washed four times with 50 mM Tris/HCl, pH 7.5 (200µl each) and subsequently eluted with 20 µl gel loading buffer (30 mM Tris, pH 6.8, 1% SDS, 1% β-Mercaptoethanol, 12.5% glycerol, 0.005% Bromphenol blue; incubation for 10 min at 95°C). Elutions were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining (**Figure 23**). Amount of PT that was bound to peptide streptavidin sepharose was calculated by densitometric evalution and plotted as a function of the amount of peptide initially used for immobilization to streptavidin sepharose (shown for pp26/9 in **Figure 23**). A maximum of PT binding was reached when 300-400 pmol peptide were used for immobilization to 1 µl streptavidin sepharose. Higher amounts of peptide did not result in higher PT binding probably reflecting effects of steric hindrance of PT.

The effectively bound fraction of peptide (pp26/9 or gurmarin/15) when an input of 400 pmol peptide was used for immobilization to 1 µl streptavidin sepharose, was evaluated by PAGE on a 12% Bis-Tris-Gel (MES running buffer) and silver staining after elution with gel loading buffer (heating at 95°C for 10 min). Amount of elutable peptide was estimated by direct comparison to defined amounts of purified PT on the same gel (data not shown): for pp26/9: 100 –150 pmol; for gurmarin/15: 50 pmol.

c. Determination of PT yield using varying amounts of sample B at constant concentration of peptide sepharose during affinity purification.

For peptide immobilization 400 pmol pp26/9 or gurmarin/15 were incubated with 1 µl streptavidin sepharose for 1 h at RT. The peptide sepharose was washed 3 times with 200 µl 50 mM Tris pH 7.5 buffer and subsequently incubated with varying amounts of Sample B (50, 66, 100, 200, 400, 600 µl, adjusted before to pH 7.0 - 7.5 by addition of HCl) for 1 hour at RT. The affinity matrices were washed 4 times with 100 µl 50 mM Tris/HCl, pH 7.5, and eluted by 4 consecutive elutions with 100 mM Carbonate buffer at pH 10.5 (each 20 µl). 5 µl

of the pooled elutions (total 80 µl) were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver staining. The amount of eluted PT was calculated on the basis of direct comparison to defined amounts of purified PT on the same gel as mass standard **(Figure 24, Table 12).**

Table 12

Input peptide 16K9 (pmol)	Input PT (pmol)	Ratio peptide: PT	Amount of PT bound (pmol)	Yield of PT relative to input amount of PT
100	300	1 : 3	~100	33%
100	200	1: 2	~88	44%
100	100	1:1	~40	40%
100	50	2:1	~24	48%
100	33,3	3:1	~16	48%
100	25	4:1	~24	96%

Input peptide 17K15 (pmol)	Input PT (pmol)	Ratio peptide: PT	Amount of PT bound (pmol)	Yield of PT relative to input amount of PT
100	300	1 : 3	~80	27%
100	200	1: 2	~64	32%
100	100	1:1	~56	56%
100	50	2:1	~16	32%
100	33,3	3:1	~16	32%
100	25	4:1	~8	32%

Input asiaolfetuin (pmol)	Input PT (pmol)	Ratio peptide: PT	Amount of PT bound (pmol)	Yield of PT relative to input amount of PT
100	200	1: 2	~8	4%
100	100	1:1	~16	16%
100	85,6	20:17	~8	9%
100	50	2:1	~8	16%
100	33,3	3:1	~8	24%
100	25	4:1	~8	35%

To compare the purification efficiencies of the peptide streptavidin sepharoses with asialofetuin sepharose a titration experiment with asialofetuin sepharose was performed in parallel under comparable conditions (same amount of affinity ligand per reaction immobilized on sepharose, corresponding to ~100 pmol affinity ligand effectively bound to the sepharose). This was accomplished by incubation of 6.85 µl of asialofetuin sepharose (batch number FA 053198: density 1.1 mg/ml, 14.6 pmol/µl) with varying amounts of Sample B (50, 66, 100, 171.3, 200, 400 µl, adjusted before to pH 7.0 - 7.5 by addition of HCl) for 1 hour at RT. Subsequently the asialofetuin sepharose was washed and bound PT was eluted and analyzed as described above. The binding efficiency of peptide streptavidin sepharose under the applied purification conditions was significantly higher than the binding efficiency of asialofetuin sepharose.

d. Reutilization of peptide sepharose for repeated PT binding and elution

To investigate the reusability of peptide loaded sepharose (pp26/9 and gurmarin/15) for repeated binding and elution of PT the sepharoses were applied for repeated cycles of PT binding, elution and regeneration (in total 4 times). For peptide immobilization 600 pmol pp26/9 or gurmarin/15 were incubated with 2 µl streptavidin sepharose over night at RT and subsequently washed 3 times with HEPES buffer. For binding of PT each peptide streptavidin sepharose was incubated with 400 µl sample B (adjusted to pH 7.0 –7.5 by addition of HCl) for 1 hour at RT and washed 4 times with 50 mM Tris/HCl, pH 7.5 (each 200 µl). PT was eluted by 4 consecutive elutions with 100 mM Carbonate buffer at pH 10.5 (each 20 µl). Subsequently the column matrices were regenerated by three washes with 10 mM HCl (1x 20 µl, 2x 100 µl) and afterwards neutralized by two washes with 200 µl 50 mM Tris/HCl, pH 7.5. This binding, elution and regeneration procedure was applied to the peptide sepharose for three additional times. 4 µl of the pooled elutions (in total 80 µl) and 7 µl of the first regeneration buffer from each binding/elution/regeneration cycle were analyzed by PAGE on 12% Bis-Tris-Gels (MES running buffer) and silver stained, indicating that the peptide sepharose may be re-utilized. **(Figure 25)**.

5. Large-scale FPLC-Purification of PT

Optimized conditions for PT binding and elution were applied for large scale FPLC purification (0.5 ml column), as shown below:

A) Immobilization of biotinylated peptide to streptavidin-sepharose: 200 nmol peptide pp26/9 were incubated for 1 h 30 min at room temperature on a rotating wheel with 1 ml 50% Streptavidin-sepharose in volume of 10 ml (HEPES-buffer). After incubation the sepharose was washed 3x with 50 mM Tris pH 7,5.

B) Binding of PT (out of sample B): The estimated amount of peptide effectively immobilized on 500 µl sepharose was 50 nmol. The peptide-sepharose was incubated with 25 ml sample B for 1 h 30 min at room temperature in a head over tail rotator (assumed concentration of PT ~ 0.5 pmol/µl, corresponding to 12,5 nmol in 25 ml, corresponding to a ratio of immobilized peptide to amount of PT of 4:1).

C) FPLC-column: After incubation the sepharose was transfered to a column (Pharmacia HR 5/5) During packing of the column the sepharose was washed with 50 mM Tris pH 7.5 (2-3 ml). Subsequently the column was taken in the flow path and washed with 20 column volumes (10 ml) 50 mM Tris ph 7,5. Immobilized PT was eluted with 11 ml 100 mM carbonate buffer pH 10.5. The elution fractions were collected in 500 µl fractions (Pharmacia Fraction Collector FRAC-100) and the elution profile was evaluated by measurement of the UV absorbance at 280 nm. After elution the column was washed with 1.5 ml 50 mM Tris pH 7,5 and subsequently regenerated with 2.5 ml 10 mM HCl followed by neutralization with 10 ml 50 mM Tris pH 7.5.

D) analysis of elution fractions and calculation of yield: The elution fractions were analyzed by PAGE (12% Bis-Tris-Gel, MES running buffer) and silver staining (**Figure 26**). Concentration of PT was determined by measuring the absorbance of the elution fractions at 280 nm (A_{280}) and comparing these results with a calibration curve prepared with purified PT (see table in **Figure 26**).

The amount of PT was additionally calculated on the basis of direct comparison to defined amounts of purified PT on the same gel as mass standard. Gel estimation leads to a yield of 8100 pmol PT. This correlates very well with the concentration determination using A_{280}. If it is assumed that 25 ml sample B contains 1125 µg of PT, more than 69%-72% is eluted of PT under these conditions. This result was verified by repetition of the FPLC run using the same peptide-sepharose after regeneration to bind PT out of 25 ml sample B. In this experiment, 803 µg PT was purified (A_{280}) (**Table 13**).

Table 13

Determination of concentration of PT in elution fraction (FPLC run #2) using A_{280}

	A_{280}	µg/ml
Elu1	0	0
Elu2	0	0
Elu3	0,091	85
Elu4	0,4185	391
Elu5	0,354	331
Elu6	0,2835	265
Elu7	0,212	198
Elu8	0,148	138
Elu9	0,0975	91
Elu10	0,0585	55
Elu11	0,0315	29
Elu12	0,025	23
Total 3-12		= 803 µg

Table 14
Summary of PT Purification Results

	Yield PT in 12x 0.5 ml fractions (6 ml)	Relative Yield versus input amount of PT (1125 µg in 25 ml) (pmol/pmol or µg/µg)	Purity
1. purification run	772 – 813 µg	69% - 72%	Comparable to PT purified on asialofetuin sepharose, 100%
2. purification run	803 µg	71%	Comparable to PT purified on asialofetuin sepharose, 100%

6. Evaluation of equilibrium and rate constants of the pp26 peptide 9 / Pertussis toxin complex formation

Equilibrium constants and rate constants for the pp26 K9 / PT complex formation were evaluated using the BIAcore 2000 instrument in HBS/EP running buffer (0,01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20) at room temperature. Binding of varying concentrations of pp26-K9 (concentartions between 2.5 nM and 100 nM) to PT immobilzed on a CM5 chip (immobilization of 6000 RU via amine coupling method) were analyzed at a flow rate of 30 µl/min. Quantitative elution of PT bound peptides were obtained by using 3 mM HCl, pH 2.5. Deducible equilibrium and rate constants were analyzed using the BIAevaluation software, the results of which are shown below:

Dissociation equilibrium constant K_D	→	7.5×10^{-9} M
Association equilibrium constant K_A	→	1.3×10^{-8} M^{-1}
Association rate constant k_{on}	→	1.3×10^{5} $M^{-1} \times s^{-1}$
Dissociation rate constant k_{off}	→	10^{-3} s^{-1}

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

CLAIMS

What is claimed is:

1. A peptide having the ability to bind pertussis toxin, the peptide being selected from the group consisting of:
RSSHCRHRNCHTITRGNMRIETPNNIRKDA (pp26-5);
RSTMNTNRMDIQRLMTNHVKRDSSPGSIDA (pp26-6);
RSNVIPLNEVWYDTGWDRPHRSRLSIDDDA (pp26-9);
RSWRDTRKLHMRHYFPLAIDSYWDHTLRDA (pp26-15);
SGCVKKDELCARWDLVCCEPLECIYTSELYATCG (G-9);
SGCVKKDELCELAVDECCEPLECFQMGHGFKRCG (G-10);
SGCVKKDELCSQSVPMCCEPLECKWFNENYGICGS (G-15); and,
SGCVKKDELCELAIDECCEPLECTKGDLGFRKCG (G-19).

2. A peptide of claim 1, wherein the peptide is:
RSNVIPLNEVWYDTGWDRPHRSRLSIDDDA (pp26-9); or,
SGCVKKDELCSQSVPMCCEPLECKWFNENYGICGS (G-15).

3. A method for generating a DNA-peptide fusion, said method comprising:

(a) covalently bonding a nucleic acid reverse-transcription primer to an RNA encoding a peptide, said reverse-transcription primer being bound to a peptide acceptor;

(b) translating said RNA to produce the peptide, the peptide being covalently bound to the reverse-transcription primer; and,

(c) reverse transcribing said RNA to produce a DNA-peptide fusion;

wherein the peptide has binding affinity for pertussis toxin.

4. A method for generating a DNA-peptide fusion, said method comprising:

(a) generating an RNA-peptide fusion;

(b) hybridizing a nucleic acid reverse-transcription primer to said fusion;

(c) covalently bonding said primer to said fusion; and

(d) reverse transcribing the RNA of said RNA-peptide fusion to produce a DNA-peptide fusion;

wherein the peptide has binding affinity for pertussis toxin.

5. A method for generating a DNA-peptide fusion comprising the steps of, in combination:

(a) providing an RNA molecule covalently bonded to a peptide acceptor;

(b) covalently bonding a nucleic acid reverse-transcription primer to the molecule of step (a);

(c) translating said RNA molecule to produce a peptide, and

(d) reverse transcribing said RNA molecule to produce a DNA-peptide fusion;

wherein the peptide has binding affinity for pertussis toxin.

6. A DNA-peptide fusion prepared using the method of claim 3, 4 or 5.

7. A peptide having affinity for pertussis toxin identified using the method of claim 3, 4 or 5.

8. A peptide of any one of claims 1, 2 or 7 wherein the peptide is biotinylated.

9. A method for purifying pertussis toxin comprising contacting a biological solution containing pertussis toxin with a peptide of at least one of claims 1, 2 or 7 bound to a solid support to form a pertussis toxin-peptide complex and isolating the complex from other components in the biological solution.

10. The method of claim 9 wherein pertussis toxin is released from the complex and isolated.

11. The method of claim 10 wherein the pertussis toxin is released from the complex by altering the pH of the environment surrounding the complex.

12. The method of claim 11 wheren the complex is exposed to a solution having an acidic pH.

13. The method of claim 11 wheren the complex is exposed to a solution having an basic pH.

14. The method of claim 10 wherein the pertussis toxin is released from the complex by altering the ionic strength of the environment surrounding the complex.

15. The method of claim 14 wherein the ionic strength is altered by exposing the complex to a solution having a high concentration of one or more ionic salts.

16. The method of claim 15 wherein the ionic salt is at least one of sodium chloride or magnesium chloride.

17. The method of claim 16 wherein the ionic salt is magnesium chloride.

18. The method of any one of claims 9-17 wherein said solid support is a bead.

19. The method of any one of claims 9-17 wherein said solid support comprises sepharose.

20. The method of claim 19 wherein the solid support consists of streptavidin sepharose.

21. The method of claim 20 wherein the peptide is biotinylated and the peptide is bound to the solid support through the interaction of the biotin moiety on the peptide and the streptavidin moiety on streptavidin sepharose.

22. A method for isolating a DNA-peptide fusion in which the peptide has binding affinity for pertussis toxin comprising the steps of, in combination:

(a) covalently bonding a nucleic acid reverse-transcription primer to an RNA encoding a peptide, said reverse-transcription primer being bound to a peptide acceptor;

(b) translating the RNA to produce the peptide, the peptide being covalently bound to the reverse-transcription primer; and,

(c) reverse transcribing the RNA to produce a DNA-peptide fusion;

(d) contacting the DNA-peptide fusion with pertussis toxin bound to a solid support to form a DNA-peptide fusion-pertussis toxin complex;

(e) isolating the complex from DNA-peptide fusions that did not complex with pertussis toxin; and,

(f) isolating the DNA-peptide fusion from the DNA-peptide fusion-pertussis toxin complex.

23. A method for identifying a peptide having binding affinity for pertussis toxin comprising carrying out the method of claim 22, and additionally determining the amino acid sequence of the peptide portion of the DNA-peptide fusion.

24. A method for identifying the DNA sequence encoding a peptide having binding affinity for pertussis toxin comprising carrying out the method of claim 22, and additionally determining the nucleotide sequence of the DNA portion of the DNA-peptide fusion.

25. An immunological composition comprising pertussis toxin isolated by the method of claim 10.

26. A peptide having the ability to bind pertussis toxin and the amino acid sequence of a peptide shown in any of Figures 3-14.

27. The method of any one of claims 3-5 or 9-24 wherein the amino acid sequence of the pertussis binding peptide includes amino acid sequences derived from gumarin or PP26.

28. The method of any one of claims 3-5 or 9-24 wherein the nucleotide sequence encoding the pertussis binding peptide includes nucleotide sequences derived from gumarin or PP26.

29. A peptide having the ability to bind pertussis toxin and comprising the amino acid sequence LGHGLGYAY.

30. A peptide of claim 29 further comprising the amino acid sequence ELAVD, ELAID, or ARWDLV.

31. A peptide having the ability to bind pertussis toxin and comprising at least one of the amino acid sequences TTASKS or KWTNEHFGT.

32. A peptide of claim 31 comprising the amino acid sequences TTASKS and KWTNEHFGT.

33. A peptide having the ability to bind pertussis toxin and comprising an amino acid sequence selected from the group consisting of
NVIPLNEVWYDTGWDRPHRSRLSIDD,
VGTTIRIAQDTEHYRNVYHKLSQYSR,
WTSMQGETLWRTDRLATTKTSMSHPP,
LSALRRTERTWNTIHQGHHLEWYPPA,
LSRLARTERTWDRIHQGHHLEWHPPA,
TMNTNRMDIQRLMTNHVKRDSSPGSI,
LSALMRTERTWNTIHQGHHLEWYPPA,
CLATRNGFVMNTDRGTYVKRPTVLQ,
CLATRNGFVQMNTDRGTYVKRPTVLQ,

35. A peptide having the ability to bind pertussis toxin and comprising the amino acid sequence XXAXRXXXXXXNTXXXXXXXXXT or XXAXRXXXXXXNTXXXXXXXXXY, where X is any amino acid.

36. A peptide having the ability to bind pertussis toxin and comprising an amino acid sequence of VXXXXXXXXDTXXXXRXXXXXLS, where X is any amino acid.

37. A peptide of any one of claims 29-36, wherein at least one amino acid is conservatively substituted.

38. A peptide of any one of claims 29-37 wherein the peptide is biotinylated.

39. A method for purifying pertussis toxin comprising contacting a biological solution containing pertussis toxin with a peptide of at least one of claims 29-38 bound to a solid support to form a pertussis toxin-peptide complex and isolating the complex from other components in the biological solution.

40. The method of claim 39 wherein pertussis toxin is released from the complex and isolated.

41. The method of claim 40 wherein the pertussis toxin is released from the complex by altering the pH of the environment surrounding the complex.

42. The method of claim 41 wheren the complex is exposed to a solution having an acidic pH.

43. The method of claim 41 wheren the complex is exposed to a solution having an basic pH.

44. The method of claim 40 wherein the pertussis toxin is released from the complex by altering the ionic strength of the environment surrounding the complex.

45. The method of claim 44 wherein the ionic strength is altered by exposing the complex to a solution having a high concentration of one or more ionic salts.

46. The method of claim 45 wherein the ionic salt is at least one of sodium chloride or magnesium chloride.

47. The method of claim 46 wherein the ionic salt is magnesium chloride.

48. The method of any one of claims 39-47 wherein said solid support is a bead.

49. The method of any one of claims 39-47 wherein said solid support comprises sepharose.

50. The method of claim 49 wherein the solid support consists of streptavidin sepharose.

51. The method of claim 50 wherein the peptide is biotinylated and the peptide is bound to the solid support through the interaction of the biotin moiety on the peptide and the streptavidin moiety on streptavidin sepharose.